STUDIES ON
CHEMICAL CONSTITUENTS
OF
VOLVARIELLA VOLVACEA (Bull. ex Fr.) Sing.
AND
OTHER EDIBLE SPECIES OF FUNGI

BY

BU-HAN HUANG

A Thesis

Summitted in Partial Fulfilment

of the Requirement for the Degree of

Master of Philosophy

June, 1982

Division of Biology

Graduate School

The Chinese University of Hong Kong

THESIS COMMITTEE

Prof. S. T. Chang

Prof. L. B. Thrower

Dr. K. H. Yung

To my parents

ACKNOWLEDGEMENTS

My appreciation is due to the Academia Sinica who sent me to study under the supervision of Professor S. T. Chang. Without the guidance, encouragement and valuable advice from Prof. Chang, my study would not have been completed. Therefore, the author wishes to express his grateful appreciation to Prof. Chang. At the same time, I would like to thank Professor L. B. Thrower for his deep concern for my study and his patient teaching. This makes me learn and advance a great deal in my two years of study. I must also thank Dr. K. H. Yung, who advised me during the project ensuring that the research went smoothly.

Thanks are also due to Mr. S. N. Mok who supplied me with many experimental materials for my research, and to Mr. Y. H. Law and Mr. M. C. Wong who analysed IR, MS, and NMR for me.

ABSTRACT

An investigation of the chemical composition of lipids of V. volvacea, L. edodes, A. bisporus, P. sajor-caju, A. auricula and T. fuciformis was the central theme of this study. The total lipid of the six edible mushrooms ranges from 0.6% to 3.1%. The sterols in lipid were separated by recrystallization and preparative TLC, and their structures were then determinated by GLC, UV, MS, and NMR. The results showed the presence of ergosterol (Provitamin D) in six mushrooms. Among them, V. volvacea was highest (0.47%), the second was L. edodes (Cracky Dongko) (0.27%) and A. bisporus (0.23%), the lowest was T. fuciformis (0.01%). The ergosterol content was higher in the matured stage (0.54%) than the egg stage (0.39%) in V. volvacea. In the two developmental stages of V. volvacea, the cap contains 0.63% ergosterol which is much higher than that of the stem (0.3%). In addition to ergosterol, L. edodes contains ergosta-5,7-dien-3β-ol, whereas V. volvacea and T. fuciformis contain ergost-7-en-3β-ol.

The fatty acids of the six mushrooms were analyzed by GLC. The results showed that they contain myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid. All of the six edible mushrooms had unsaturated fatty acids as their chief fatty acids. The

unsaturated fatty acids of V. volvacea were as high as 83.3% of the total fatty acid content, whereas that of L. edodes (Cracky Dongko) was 75.9%, A. bisporus 74.1%, P. sajor-caju, 76.6%, A. auricula, 73.1% and T. fuciformis 69.2%. It is thus clear that the characteristic of fatty acid in the edible mushrooms was their chief by unsaturated fatty acid content. Linoleic acid has been claimed to be a kind of essential fatty acid. The content of linoleic acid in these edible mushrooms ranges from 27.98 to 76.25% of the total fatty acids. L. edodes (Cracky Dongko) had the highest content at 76.25%, the next was V. volvacea at 69.91%, and the lowest content was T. fuciformis at 27.98%. The lipid content of the fungus is clearly related to developmental stages and parts. For example, in V. volvacea, the total lipid content is higher in the mature stage (3.6%) than in the egg (2.6%). Moreover, in the mature stage, the linoleic acid content of the stem makes up 82.41% of the total fatty acids but of the cap only 57.42%; in the egg the values are stem 85.46% and cap 78.56%. From the above, it can be concluded that the mushrooms contained more of the essential fatty acid and ergosterol which are very beneficial to mankind.

The flavour substance of V. volvacea ranges from 0.001 to 0.0016%. According to the peaks in the GLC analysis, the flavour substance is very complicated. One fraction

of acidic flavour substance can be separated by sodium bicarbonate solution. This extract is a very strong flavour substance. The chloroform extract of V. volvacea contains much triterpenoid, the acidic aqueous extract contains alkaloid and the methanol extract contains cardiac glycoside. Some of them are biologically active substances, and their presence in V. volvacea requires further investigation.

TABLE OF CONTENTS

Chapter 1

INTRODUCTION

In the past, there were many myths and legends about the edible mushrooms in the oriental countries. For example there was a legend that the mushroom would bring longevity and would make us never grow old. This reflects the fact that even in ancient times, people regarded edible mushrooms as precious substances. China has recognised and used the true fungi for four thousand years(Wang, 1981). As a drug, mushrooms had been mentioned in the old medical books of China. Li Chik-Chen's "Pen Tsao Kang Mu"(the classic Chinese materia medica) recorded that Lentinus edodes(Berk.) Sing. and Auricularia auricula(L. ex Hook.)Underw. can cure many diseases(Yang and Chao, 1981). Through this, we can see that people have known how to use the mushroom in nature for a long time.

Mushrooms are a delicious food and they can be used medically, but the natural sources of mushrooms are limited. Therefore, they have gradually been cultured by man. According to "Huang Jeng Shu Nurng" of Tong Dynasty of China Castanopsis Spach was used in culture of Lentinus edodes; the method was described in detail. This is very likely the earliest recorded method of culture and is earlier than the culture of Agaricus bisporus(Lange) Imbach in A. D. 1700 by a thousand years. China(in the south eastern

provinces) has two hundred years experience in mass culture of the straw mushroom Volvariella volvacea(Bull. ex Fr.) Sing.as a daily vegetable(Chang, 1977). The method was soon dissiminated to Korea, Japan and the Southeast Asian countries(Park, et al., 1974; Gruen, 1964; Vicencio, 1916). Nowadays, the culture of some edible mushrooms in some countries has turned into a large-scale commercial enterprise. According to statistics in 1979, the worldwide production of edible mushrooms had reached 1,210,000 tonnes(Chang, 1981), and it is believed that the amount would be far exceeded now. The reasons for the rapid development of the culture of the edible mushroom are the exploding population and the limitation of food resources. Gradually, people have begun to utilize industrial wastes, agricultural wastes and even domestic wastes like straw, spoiled wool, sawdust etc. as the culture media. The edible mushrooms produced from these materials contain 30~50% protein(of dry material) and a large amount of mineral salts and vitamins. Thus these mushrooms have a high nutritive value and are good for our health. Success in mass production of mushrooms would then be a short cut to solving the deficiency of protein in the underdeveloped countries. Therefore, the investigation and culture of the edible mushroom are promoted by the World Organizations, e.g. United Nations Educational, Scientific and Cultural Organization(UNESCO), and the United

Nations University(UNU). The organizations support and finance relevant projects and hence the production of edible mushroom is enhanced.

With the increase in the amount of the edible mushrooms, we then face a lot of questions that people are concerned with. What are the chemical constituents of these mushrooms? What is their particular nutritive value? How effective would they be as drug? All these questions are very appealing. There are many reviews about the nutritive value of edible mushrooms(Crisan and Sands, 1978; Cheng, 1979; Ogundana and Fagade, 1981; Khana and Garcha, 1981). However, all these reviews are only based on the proximate composition: crude protein, crude lipid, crude cellulose, carbohydrate, etc.. That the water extract of edible mushrooms contains antivirotic was found in the 1960's. This initiated more research concerning the chemical constituents of Lentinus edodes as an anti-tumour substance(Cochran, et al., 1960). Through this action, the investigation of Lentinus edodes was more intensified.

Research on the chemical constituents of the unique pleasant flavour of edible mushrooms was begun in the early 1930's. Nevertheless, up till now, the list of the chemical constituents of the edible mushrooms is still meager. Being a kind of natural compound as mushrooms are, the chemical constituents are very complicated. The complexity is increased because there are so many species. Even in

Lentinus edodes, the chemical composition remains unclear. It is no wonder that while there are many publications about edible mushrooms, there are none which specifically list the chemical constituents. Professor S. T. Chang in the introduction of his book "The Biology and Cultivation of Edible Mushroom" had emphasised that "First, more extensive chemical analysis should be carried out, subjecting various components to a well-planned test in an attempt to assay their nutritional value. These data are important since they can provide us with a basis for objectively evaluating any edible mushroom."

In the past, there has been little research on the chemical constituents of the edible mushrooms. This is because people neglected the importance of the edible mushroom, and they were limited by the technology. Today, thin layer chromatography(TLC), gas liquid chromatography(GLC), mass spectroscopy(MS) and nuclear magnet resonance(NMR) methods are widely applied new technologies. Through the use of these technologies, it is then possible to analyse the special chemical constituents in the mushroom. We have applied the above technologies on Volvariella volvacea, Lentinus edodes, Agaricus bisporus, Pleurotus sajor-caju(Fr.) Singer, Auricularia auricula and Tremella fuciformis Berk. We have isolated and identified the fatty acid and sterols in these mushrooms. At the same time, we have compared the

fatty acids and sterols of different parts and different developmental stages of these mushrooms. Among these, the fatty acids and sterols in Volvariella volvacea, Pleurotus sajor-caju and Tremella fuciformis have not been mentioned in any previous report. Therefore the data in this thesis not only illustrate clearly the untritive values of several common edible mushrooms, but also provide a more detailed scientific background of these mushrooms.

This thesis includes three parts:

Part 1 General aspects, comprising two chapters. Chapter 1 is the introduction, and chapter 2 is the general review.

Part 2 A consideration of the fat-soluble fraction which consists of three chapters. Chapter 3 is a study of the separation and identification of the sterols in mushrooms. Chapter 4 is a study of the separation and identificarion of fatty acids in mushrooms. Chapter 5 is a description of flavour composition and other compounds.

Part 3 General summary and conclusion.

Chapter 2

GENERAL REVIEW

2.1 BIOLOGICAL ASPECTS OF EDIBLE MUSHROOMS

At present the deveolpment of the culture of editle mushroom is becoming more intensified: on one hand, people extend the species and the areas to be cultivated; on the other hand, they try to high yield and automation direction. Hence, the understanding and good grasp of the biological characteristics and technique of culture become an important issue in the further development of the cultivation of the mushrooms. Luckily, these techniques have been descanted. Detail descriptions have been found in "The Biology and Cultivation of Edible Mushrooms"(Chang and Hayes, 1978), "The Biological Basis of Edible Fungi"(Yang, 1980), "Nutritional studies on Volvariella volvacea"(Quimio, 1981) and "Tropical Mushrooms-Their Biological Nature and cultivation Methods" (Chang and Quimio, 1982).

Because all fungi lack chlorophyll, they cannot, like the green plants, get their energy directly from the sun. Instead, they must obtain their nutrients from preformed cellulosic and woody waste materials. Like all filamentous fungi, the mushroom hypha can grow over, into, or through the substrate by extension of the hyphal tip; the older hypha is not capable of growth, but it has an important role in supporting growth of the tip and development of

fruiting bodies absorbing and transporting nutrients to the active growing apices. Mushroom hyphae liberate large amounts of extracellular cellulase and related types of enzymes to help degradation of the many types of macromolecules, such as cellulose,hemicellulose, lignin, protein etc. present in the substrate. The simple, soluble smaller molecules which result are then absorbed by the fungal cells. Due to these characteristics, mushroom hyphae can easily colonize the substrate and absorb the nutrients from the substrate for growth(Chang, 1981).

The development and growth of edible mushrooms require different temperature, moisture, nutrition, oxygen, pH and light for different species. For example, the hyphae of Volvariella volvacea grow best at 35°C, Auricularia auricula at 30°C and Agaricus bisporus at 24°C. Even in the same species, the different stages of development require different optimal conditions. Generally speaking, the growing stage of hyphae require a higher temperature than the fruiting-body formation stage. In the development stage of fruiting body, adequate oxygen supply and high relative humidity are required other than mass nutrients, water and certain other specific conditions. The requrements of several species of the edible mushrooms with regard to temperature, humidity and pH for growth and development are recorded in "The Biology and cultivation of Edible Mushrooms(Chang and Hayes, 1978).

To become a new scientific subject, the cultivation of edible mushrooms was developed from disciplines of microbiology, fermentation and environmental engineering. In order to improve the traditional cultivation technique, more powerful techniques like mutation breeding, protoplast fusion and cell hybrids have been introduced in breeding programmes for high yield and good quality. Beginning with Humfeld(1948) reporting the application of liquid culture of hyphae and the more recent report of Terey(1968) reporting the details of the submerged culture, the culture of edible mushroom has moved from agriculture into an industrial age. By this mean, mass hyphae of edible mushroom and their metabolites can be produced within a very short period of time, for example, an fermentator can produce several tonnes to hundred tonnes within ten days. This high efficiency of biomass production is incomparably greater than the traditional agricultural production.

Improving methods in the mushroom workshop: changing the manual operation of compost, inoculation, water spraying, maintenance and harvesting into an automatic operation are other aspects of industrialization of the edible mushroom cultivation.

All in all, no matter which area is to be promoted in the industrialization of edible mushroom cultivation, research on the biology of edible mushroom remains a very

important basis for them. This is the reason why we emphasise it repeatly and strongly.

2.2 NUTRITIVE COMPOSITION OF EDIBLE MUSHROOMS

The nutritive composition of edible mushrooms usually includes proteins, carbohydrates, fat, vitamins and minerals etc.. this composition can be used to assess the nutritive value of edible mushroom. In the past, this was based on the proximate composition because the method of determination is simple and fast. Though the proximate composition cannot reflect completely the composition of the edible mushrooms, yet it can illustrate the nutritive value of the mushroom in general. Therefore, the proximate composition of several mushrooms has been analysed(Tables 2.1 and 2.2).

2.2.1 Protein and amino acid

Among macromolecules, protein appears to be the most complicated in the living organism. In plants, the content of protein is low in comparison with that of carbohydrates. However, in Volvariella volvacea and Agaricus bisporus. the protein content reaches 25-35% (Tables 2.1 and 2.2), so that, they are good protein sources.

Usually protein quantity is expressed in terms of crude protein content which is calculated from the nitrogen content using the conversion factor(N X 6.25) based on the presumption

that most protein contains 16% nitrogen, that they are approximately 100% digestible, and that negligible amounts of non-protein nitrogen are present(Crisan and Sands, 1978). The figure for total protein obtained by this method is obviously higher than the actual one. In order to account for the presence of non-protein nitrogen in mushrooms, the crued protein content of mushroom is colculated from the nitrogen content using the conversion factor(N x 4.38), assuming that mushrooms are 70% digistible(70% N X 6.25) or (N X 4.38) (Crisan and Sands, 1978). At present the most accurate method is the assay of total amino acid in the edible mushroom(or protein amino acid only).

Overall, proteins contain some tweenty amino acids, and those contained in some proteins of edible mushrooms are listed in Table 2.3. Amino acids may be classified as essential(which cannot be synthesized by man) or non-essential. Foods differ in their content of essential amino acids: for example meat and fish proteins usually have all of them, whereas most cereal and vegetable proteins lack some of them. Mushroom proteins is one of the best in this respect(Edwards, 1975). Besides those mentioned in Table 2.3 some edible mushrooms contain unusual amino acids, like the sacchraropin from L. edodes and tricholomic acid from Tricholoma muscarium. These unusual amino acids have biological activity, so that further studies on the amino acids of edible mushrooms are important.

2.2.2 Fatty acid and sterols

In mushrooms the crude fat includes representatives of all classes of lipid compounds including free fatty acids, mono-, di- and triglycerides, sterols, sterol esters and phospholipids(Crisan and Sands, 1978). The fat content of edible mushrooms varies greatly among species(from 0.6-8%). The fatty acids in the saponifiable material of some mushrooms, after hydrolysis of the fat, are shown in Table 2. 4. The fatty acids in the edible mushrooms are mainly unsaturated, and include linoleic acid which cannot be synthesized by human beings. Besides the nutritive needs, linoleic acid, as pointed out by Chu(1980), possesses particular physiological function in the prevention of arteriosclerosis and has a hypocholesterolemic effect.

One of the characteristics of the edible mushrooms is the possession of a particular high concentration of unsaponifiable material, which exceeds 50% in some example. It had been proved that these unsaponifiable materials contain large amounts of ergosterol which occurs in concentrations of 0.2-0.4% dry weight(Sum, 1933; Oka, et al., 1973; Yoshicla, et al., 1979). Ergosterol is a kind of Provitamine D, and the amount of ergosterol thus can be used as index of the nutritive value.

The fat content of edible mushrooms ranges 2 to 8 percent. If we set aside the oil plants such as peanut, sesame and bean, the fat content of edible mushrooms is highest among

the cereals and vegetables. In comparison with animal meat, the fat content of edible mushroom is low. However, the fat of animal meat is mainly composed of saturated fatty acids and large amounts of cholesterol, which are considered to be undesirable dietary components. On the contrary, the fat of edible mushrooms are mainly composed of unsaturated fatty acids. Both these unsaturated fatty acid and ergosterol, as mentioned above are basic necessities for life. Unsaturated fatty acids, moreover, can prevent obesity and arteriosclerosis.

2.2.3 Carbohydrate and fibre

Fresh mushroom contains relatively large amounts of carbohydrate and fibre. The carbohydrate content in mushroom may consist of a large variety of compounds(Crisan and Sands, 1978). Some edible mushroom contain pentose(xylose and ribose), methyl pentose(rhamnose and fucose), hexoses(glucose, galactose and mannose), disaccharides(sucrose), amino sugars (glucosamine and N-acetylglucosamine), sugar alcohols(mannitol and inositol) sugar acids(galacturonic and glucuronic acids) as well as unidentified uronide and methyl sugars (McConnell and Esselen, 1974; Hughes, wt al.,1958).

Polymeric carbohydrates which occur include glycogen (McConnell, et al., 1974) and chitin; the latter is a major constituent of the fibre content. $\alpha\alpha$-Trehalose is a disaccharide that occurs in all edible mushrooms(Birch, 1963, 1973).

After hydrolysis, the disaccharide and polysaccharide will break into monosaccharide. It is well known that the monosaccharide generates energy after oxidation. Carbohydrates often do not arouse people's attention merely because it is easily available and cheap to be obtain. However, in mushroom, there are amino sugars, sugar alcohols and polysaccharides with special biological activity. Some of these can increase an immunity to infection and are whole good to our health.

2.2.4 Vitamines and minerals

Mushrooms appear to be good sources of several vitamines including thiamine, riboflavin, niacin, biotin, and ascorbic acid(Anderson and Fellers, 1942; Gilbert and Robinson, 1957). Recently, Hayes and Hand(1981) found in sporophores of A. bisporus an average around 0.4-0.7 μg vitamine B_{12} per g dry matter. Vitamine A(retionol) activity is relatively uncommon although several mushrooms contain detectable amounts of pro-vitamine A measured as the β-carotene equivalent.

Mushrooms probably contain every mineral present in their growth substrate(Crisan and Sands, 1978). In general, mushrooms contain significant quantities of phosphorus, sodium and potassium, a lesser amount of silicon, aluminium, magnesium, and a very low content of iron, sulfur and chlorine(Gilbert and Robinson, 1957; Crisan and Sands, 1978).

2.2.5 Nutritive value of edible mushrooms

In the past there has been disagreement about the nutritive value of mushrooms. This was largely because evaluation was made difficult because nutritive changes withs such factors as the age or developmental stage of the sporophores, the substrate on which the crop was raised, and certain intrinsec compositional variabilities(Kurtzman, 1975; Flegg and Maw, 1976).

Thus, the composition of edible mushrooms, like other natural products, changes with various factors. However, we now know under which conditions a high production of best quality edible mushrooms will be produced. Edible mushrooms, like other agricultural product, cannot give the same proximate composition every time, but at least we can get a relatively stable index. In addition to the advanced culture techniques and automatic production, the quality of the mushroom produced will be improved, and so made into a good food resource.

In the absence of animal feeding studies, the most reliable method of estimating the nutritive value of mushrooms appears to be a measure of the content of essential amino acids(Daniel and Lee,1981), and several methods can be used for determining the nutritional value of foods based on their content of essential amino acids. Crisan and Sands (1978) proposed the use of a nutritional index(NI) calculated as:

$$\text{Nutrition Index} = \frac{(\text{essential amino acid index} \times \text{percentage protein})}{100}$$

where the essential amino acid index is calculated as a ratio of the essential amino acids contained in a particular food relative to the essential amino acid pattern based on known adult human dietary requirements.

The nutritional indices for several foods in comparison with two species of mushrooms are presented in Table 2.5 A. bisporus ranks above all vegetables except soybeans and spinach. Even the less nutritive shiitake mushroom ranks above corn, potatoes, turnips, tomatoes and carrots in its NI score. A. bisporus can therefore be considered to provide nutritive value comparable to several high protein foods. Between 70 and 90% of the protein present can be easily digested from mushrooms.

Khaua and Garcha(1981) used the combination of chemical evaluation and biological evaluation to estimate the different nutritive indeces of the edible mushrooms. We think that this is right procedure. The biological evaluation is based on proximate composition and chemical constituents. The proximate and chemical constituents of some edible mushrooms are therefore listed in Tables 2.1-2.5.

Apart from high protein content, good quality of fat and particular carbohydrates, edible mushrooms are good resources of vitamines and mineral salts.

Table 2.1 Proximate Composition of Edible Mushroom(fresh)

Units: (1) Moistire: g/100g fresh sample, (2) Energy value: kcal/100g dry sample

(3) Other: g/100g dry sample

Species	Moisture	Crude Protein (N X 4.38)	Crude Fat	Carbohydrate		Crude Fibre	Ash	Energy value
				Total	N-free			
Agaricus bisporus	78.3-90.5	23.9-34.8	1.7-8.0	51.3-62.5	44.0-53.5	8.0-10.4	7.7-12.0	328-368
Agaricus campestris	89.7	33.2	1.9	56.9	48.8	8.1	8.0	354
Boletus edulis	87.3	29.7	3.1	59.7	51.7	8.0	7.5	362
Calmmulina velutipes	89.2	17.6	1.9	73.1	69.4	3.7	7.4	378
Lentinus edodes	90.0-91.8	13.4-17.5	4.9-8.0	67.5-78.0	59.5-70.7	7.3-8.0	3.7-7.0	387-392
Pleurotus ostreatus	73.7-90.8	10.5-30.4	1.6-2.2	57.6-81.8	48.9-74.3	7.5-8.7	6.1-9.8	345-367
Volvariella diplasia	90.4	28.5	2.6	57.4	40.0	17.4	11.5	304
Volvariella volvacea	88.0-88.4	29.5-30.1	5.7-6.4	50.9-60.0	39.0-49.6	10.4-11.9	4.8-12.6	374-338

Source: Crisan and Sands(1978)

Table 2.2 Proximate Composition of Edible Mushrooms(dry)

Units: (1) Moisture: g/100g dry sample, (2) Energy value: kcal/100g dry sample

(3) Others: g/100g dry sample

Species	Moisture	Crude Protein	Crude Fat	Carbohydrate	Crude Fibre	Ash	Energy Value
Agaricus bisporus	9.0	36.1	3.6	31.2	6.0	14.2	302
Lentinus edodes	18.9	13.0	1.8	54.0	7.8	4.9	284
Auricularia auricula	10.9	10.6	0.2	65.5	7.0	5.8	306
Tremella fuciformis	10.4	5.1	0.6	78.3	2.6	3.1	339

Source: Yang(1981)

Table 2.3 Amino Acid Composition of Some Edible Mushroom

Unit: mg amino acid /g corrected crude protein nitrogen

a	Agaricus bisporus	Agaricus bisporus	Agaricus bisporus	Boltus edulis	Lentinus edodes	Morchella esculenta(stipe)	Morchella esculenta(pileus)	Pleurotus ostreatus	Pleurotus ostreatus	Volvariella diplasia	Egg reference protein	FAO reference protein
Ile	366	200	214	93	218	420	259	266	267	491	340	250
Leu	580	329	348	378	348	429	384	390	610	312	540	440
Lys	527	400	357	611	174	375	339	250	287	384	440	340
Met	126	41	71	192	87	72	62	90	97	80		
Cys	71	47	80	1041	nd[b]	0	27	29	29	205		
Phe	340	186	277	331	261	268	232	216	233	437		
Tyr	286	171	89	388	174	187	161	184	189	143		
Thr	366	243	250	342	261	277	268	264	290	375	290	250
Trp	143	91	nd	756	nd	nd	nd	61	87	98	110	60

Continued overleaf

Table 2.3 (Continued)

	Agaricus bisporus	Agaricus bisporus	Agaricus bisporus	Boletus edulis	Lentinus edodes	Morchella esculenta (stipe)	Morchella esculenta (pileus)	Pleurotus ostreatus	Pleurotus ostreatus	Volvariella diplasia	Egg protein	FAO protein
Val	420	112	304	254	261	357	366	309	326	607	410	310
Arg	446	529	268	823	348	134	286	306	334	366		
Ala	473	414	420	544	305	393	411	450	403	nd		
Asp	821	400	379	544	392	429	491	564	470	nd		
Glu	1107	629	813	803	1349	768	786	890	1041	nd		
Gly	366	229	250	321	218	330	357	273	281	nd		
Pro	366	457	250	476	218	268	339	269	287	nd		
Ser	393	243	241	316	261	214	250	271	309	nd		
Total essential amino acids	3225	1820	1990	4386	1784	2384	2098	2059	2415	3685		
Total amino acids	7376	4841	4607	8933	4962	5125	5018	5169	5747	nd		

Source: Crisan and Sands(1978)

(a) Ile(isoleucine); Leu(leucine); Lys(lysine); Met(methionine); Cys(cystine);Phe(phenylalanine); Tyr(tyrosine); Thr(threonine); Trp(tryptophan); Val(valine); Arg(arginine); His(histidine); Ala(alanine); Asp(aspartic acid); Glu(glutamic acid); Pro(proline); Ser(serine).

(b) Not determined

Table 2.4 Total Fatty Acid Composition of Some Edible Mushrooms

Species	Fatty Acids(% of Total fatty acid)					
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Lentinus edodes	0.4	16.8	1.0	0.9	2.4	77.8
Agaricus bisporus	3.7	9.2	3.0	6.3	4.1	59.2
Volvariella volvacea*	0.5	10.5	0.6	3.5	12.7	69.9

Source: Yoshida(1979), Holtz and Smith(1979), * our data.

$C_{14:0}$ myristic acid $C_{16:0}$ palmitic acid $C_{16:1}$ palmitoleic acid

$C_{18:0}$ stearic acid $C_{18:1}$ oleic acid $C_{18:2}$ linoleic acid

Table 2.5 Comparison of Nutritive Value of Two Species of Mushrooms with Foods

Food	Nutritional Indices
chicken	59
beef	43
pork	35
soybeans	31
spinach	26
milk	25
Agaricus bisporus	22
kidney beans	21
peanuts	20
cabbage	17
cucumbers	14
Lentinus edodes	13
corn	11
turnips	10
potatoes	9
tomatoes	8
carrots	6

Source: Daniel and Lee(1981)

2.3 THE MEDICAL EFFECTS OF EDIBLE MUSHROOM AND THEIR ACTIVE PRINCIPLES

What attracts many peoples' attention to the medical effects of edible mushrooms is their anti-tumour and hypocholesterolemic effects. These will now be considered.

2.3.1 Anti-tumour effect of edible mushroom

2.3.1.1 General anti-tumour effects

Numerous papers had been published concerning the anti-tumour effect of polysaccharides and zymosan before the anti-tumour effect of mushrooms was reported(Roe, 1959; Creech and Breuninger, 1962; Sakai, et al., 1964). It was found that the polysaccharide extracted from mushrooms exhibited a higher activity against tumours. Doses of 5-30 mg/kg is enough to obtain a remarkable anti-tumour effect in mice with an inhibition ratio of 100% and very low toxicity(Chihara,1969). Japanese scientists also began to study the effective substance from species of Basidiomycetes and significant results have been obtained (Chihara, et al., 1970; Sasaki, et al., 1971).

2.3.1.2 The effective components

The method for separating effective components and its results are shown in Figure 2.1 and Table 2.6 respectively. Recently, the methods of extraction and isolation by the

Korean workers(Kim, et al., 1979; Chung, et al., 1980) were: 200g of material was homogenized and extracted with 0.1N NaOH by refluxing for 7 hours. After filtration, the residue was extracted with 0.1N and 0.4N NaOH by refluxing for 3 and 2 hours respectively. The filtrates were mixed together and evaporated under reduced pressure to 700 ml. It was dialysed at 5°C for 72 hours and then centrifuged until all suspended material was removed. The supernatant was lyophilized and a product was obtained for animal tests.

The effective component of the isolated product was confirmed by means of paper chromatography(PC), TLC, GLC, UV, IR, and was found to a polysaccharide, named "lentinan".

It was previously reported that the effective component of the mushroom extract was a kind of polysaccharide which contained the elements of carbon, hydrogen and oxygen but no sulfur or nitrogen. Later, a protein which was found to have anti-tumour activity was isolated from a mushroom. It was further found that the RNA of spores of Collybia velutipes(Curt.) Quel. had a stronger anti-tumour effect than the polysaccharide(Suzuki, et al., 1974). Recently, South Korean scientists working on pharmacology have reported that the active anti-tumour components were macromolecules built up by polysaccharieds and proteins(Park, et al., 1979).

From the above discussion, it can be seen that the polysaccharide is not the only substance responsible for

the anti-tumour effect of mushrooms. Moreover, the effective anti-tumour components are not confirmed to a particular kind of mushroom. These facts suggest a more detail study on the effective anti-tumour components of mushrooms is necessary.

2.3.1.3 The mechanisms of action of effective anti-tumour components

At present, most scientists believe that the effective components bring about their anti-tumour effect by stimulating the immune respone or by acting as an interferon inducer (Maeda, et al.,1971; Hamuko, et al., 1971; Tsumoda and Ishida, 1970). Interferon is used as a medicine in treating tumours. During the extraction of anti-viral substance from mushroom, it was found that the extracted substance possessed some properties similar to that of interforon. For example, they were effective in cells only, stable to acid and heat, and sensitive to trypsin.

In general, it is indisputable that mushroom do contain a certain kind of effective anti-tumour component. However, the nature of these effective components and their mechanisms of action are still not well understood and further studies are required.

2.3.2 Effect of edible mushroom on cholesterol level

2.3.2.1 Hypocholesterolemic activity

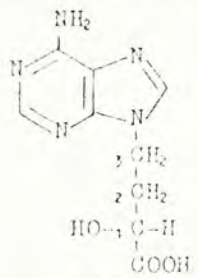
For many years it has been said that mushrooms were effective in curing heart disease, but systematic studies were not started until the 1960's when Japanese scientists began to work on it. They successfully isolated an active principle of alkaloid from aqueous extract of L. edodes and named it "eritadenine"(Kaneda, et al., 1963; Tokuda and Kaneda, 1964; Kaneda and Tokuda, 1966; Tokita, et al., 1971; Tokuda et al., 1971).

The eritadenine or ground, dried mushroom was added to the diet of rats. After a period the rat's plasma cholesterol level were found reduced. Besides L. edodes. some other species such as A. bisporus, Auricularia polytricha were also screened, and among them, the effect of A. bisporus was similar to that of L. edodes, under the same experimental conditions, the plasma cholesterol level of the Lentinus-fed group and of the Agaricus-fed group were respectively 99.6 mg/100 ml and 117 mg/100 ml, with the control group at 185 mg/100 ml. This result indicates that a diet containing 5% of ground, dried mushroom(and particularly of the caps) can markedly reduce the plasma cholesterol level, suggesting that the effective components have a very high activity.

2.3.2.2 Active principle

Extraction, separation and chemical structure of effective

component had been described by Saito(1970), and the structure of eritadenine is shown below(Kamiya, et al., 1972):



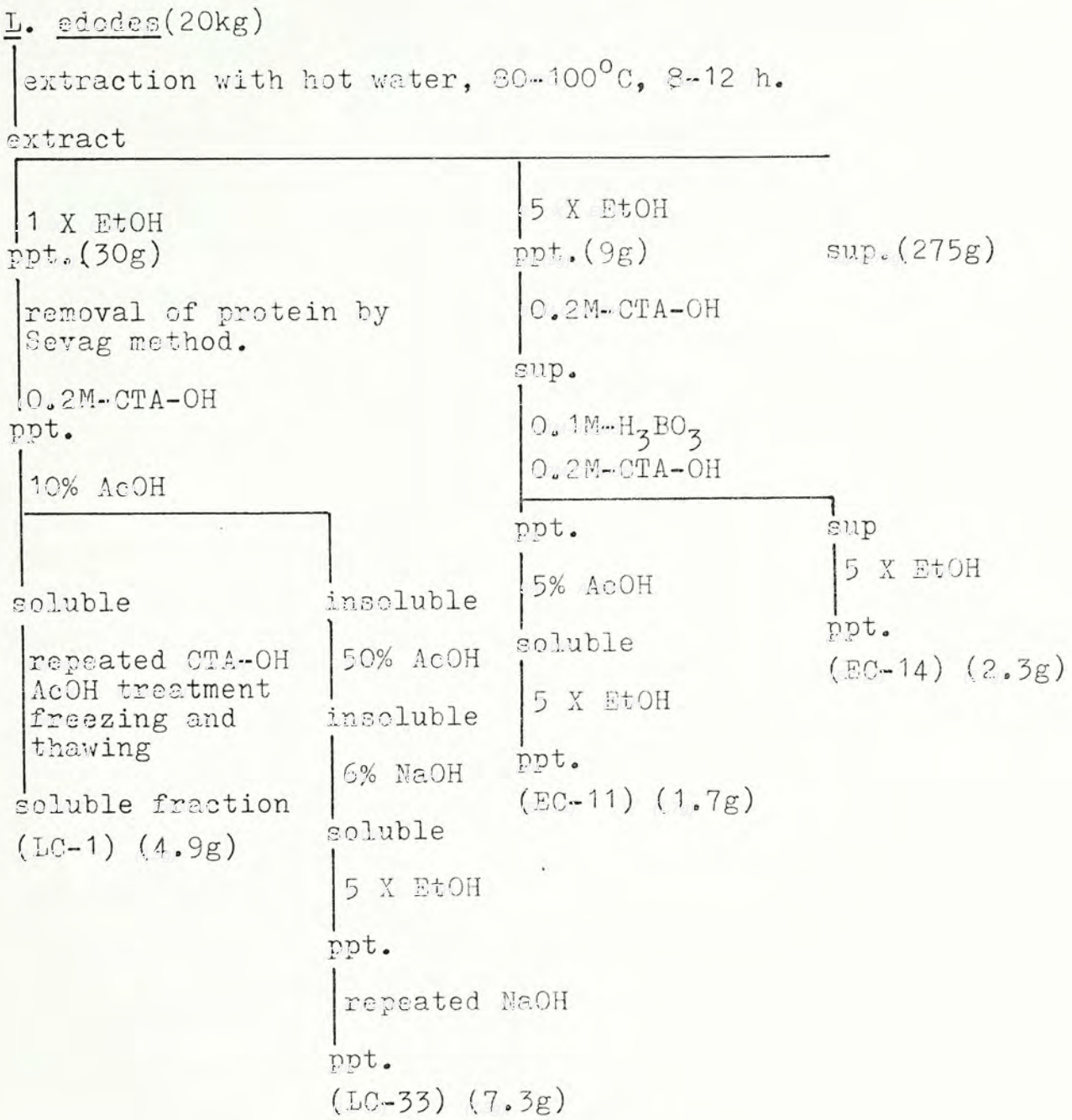
Besides eritadenine, deoxyeritadenine and 4-(6-amino-9H-purin-9-yl)-propionic acid were also found in the extract of L. edodes(Saito, et al., 1970). Their derivatives have been applied in clinical practice to treat arteriosclerosis.

2.3.2.3 Mechanism of reducing cholesterol level

It has been learned that eritadenine or L. edodes could reduce plasma cholesterol through the following mechanism(Tokuda, et al., 1974):

1) Inhibition of endogenous synthesis of cholesterol.
2) Promotion of the catabolism from cholesterol to bile acids.
3) Inhibition of reabsorption of cholesterol.
4) Stimulation of the excretion of cholesterol.

Fig. 2.1 Fractionation of Polysaccharides from L. edodes



Source: Chihara, et al.(1969)

Table 2.6 Anti-tumour Activity of Polysaccharide Preparations from L. edodes on Mice Bearing Sarcoma-180

Sample	Dose	Body weight change	Average weight of tumour	Inhibition ratio	Complete regression
LC-1	100 mg/kg	+4.7g	2.1g	71.3%	2/9
	Control	+1.3	8.1		0/10
	30 mg/kg	-1.2	0.2	96.5%	8/9
	15 mg/kg	+2.0	0	100%	10/10
	5 mg/kg	+1.9	0.04	99.3%	8/10
	Control	+3.0	5.7		0/10
	30 mg/kg	+1.0	0	100%	10/10
	5 mg/kg	+2.4	0.3	96.0%	9/10
	Control	+4.7	7.5		0/10
LC-33	25 mg/kg	+1.8	1.7	73.0%	2/9
	5 mg/kg	+1.2	0.15	97.5%	7/10
	1 mg/kg	+2.7	0.31	95.1%	6/10
	Control	+6.0	6.3		0/10
	5 mg/kg	+2.2	0.2	97.3%	7/10
	200 mg/kg	+4.1	1.6	78.1%	6/10
	Control	+6.7	7.3		0/10
EC-11	50 mg/kg	+2.1	6.2	-1.6%	0/10
	Control	+5.9	6.1		0/10
EC-14	50 mg/kg	+3.0	4.4	42.1%	0/10
	Control	-0.1	7.6		0/10

Source: Chihara, et al.(1969)

2.4 FLAVOUR AND DELICACY COMPOUNDS OF EDIBLE MUSHROOM

2.4.1 Flavour compounds of edible mushroom

Flavour and delicacy are two different qualities. Their chemical compositions are entirely different. However, since the best edible mushrooms usually have both qualities Most people have an impression that they are one. Generally speaking, the chemical constituent of delicacy is water-soluble whereas that of flavour consist of both a water-soluble component and a fat-soluble component. The chemical constituents of flavour are complicated and they are present only in parts per million(Pyysalo, 1979). Although work has been done in isolating these chemical constituents, it is only recently, owing to the application of GC and GC-MS, that the chemical constituents of flavour have been analysed to a preliminary extent.

The usual way of extraction of flavour compounds is by means of steam distillation and solvent extraction. In recent years, the application of headspace which can effectively collect the low boiling point constituents had been extended(Yajima, et al., 1981). Irrespective of the method, the amount flavour substance extracted is very small. Since it is present only in parts per million analysis is difficult and only very few compounds have been isolated. Most of them are identified via the combined utilization of GC and GC-MS. Some of the identified flavour compounds of

edible mushrooms are shown in Table 2.7, but it must be noted that these compounds may change during drying or cooking

The compounds believed to be responsible for the flavour of fresh, wild mushrooms are mainly the carbonyl compounds and alcohol constituents. 1-octen-3-ol was the main volatile compound in all the mushrooms and is the most important compound for flavour and aroma(Pyysalo,1979). This and other compounds are listed in Table 2.8. In addition, L. edodes has four other compounds that contain sulphur: 1,2,3,5,6-pentathiepane, 1,2,3,4,5,6-hexathiepane, 1,2,4,6-pentathiepane, 1,2,4-trithielane(Morta and Kobayashi, 1966). Thence, the overall flavour of edible mushrooms is due to a large set of compounds that interact with, and counter-balance, one another.

2.4.2 Delicacy compounds of edible mushroom

As food, the most appealing feature of edible mushroom is its delicacy, and it has been found that this is due to 5'-GMP, together with certain amino acids and some poly-saccharides. Their interaction creates the particular delicacy of edible mushroom(Hatanaka, 1974). 5'-GMP is formed by the action of RNAase on ribonucleic acid. This explains why the recently harvested mushrooms do not have maximum delicacy and after submergence in cold or hot water the delicacy will increase, the later treatments, more 5'-GMP

is foumed. The different types of nucleotide formed in L. edodes and A. bisporus via different means are shown in Table 2.9. 5'-GMP is an monosodium glutamate enriching agent in the food industry. The addition of 5'-GMP to monosodium glutamate would turn it into supermonosodium glutamate, and thus increase the delicacy several hundred fold.

Table 2.7 Summary of the Volatile Compounds Identified in Some Mushrooms

Compound	Compound
Carbonyl compounds	2,4-nonadienal
acetaldehyde	2,4-decadienal
3-methylbutanal	2,4-undecadienal
2-pentanal	cinnamaldehyde
pentanal	6-methyl-5-heptene-2-one
2-hexanone	acetophenone
2-methyl-2-penten-4-one	Esters
hexanal	formate
heptanone	acetate
4-methylcyclo-hexanone	propionate
3-octanone	ethyl benzoate
2-octanone	benzyl acetate
octanal	methyl decanoate
1-octen-3-one	5-hydroxyheptanoic acid lactone
trans-2-octenal	4-hydroxynonanoic acid lactone
benzaldehyde	4-hydroxydecanoic acid lactone
2-decanone	n-butyl formate
phenylacetaldehyde	methyl hexanoate
butyrophenone	ethyl propionate
3,3,5-trimethyl-cyclohexanone	
isophorone	ethyl 2-methylbutyrate

Continued overleaf

Table 2.7(Continued)

Compound	Compound
hexyl acetate	Alcohols
cis-3-hexenyl acetate	isobutanol
methyl 2-octenoate	butanol
bornyl acetate	3-methylbutanol
methyl 2,4-decadienoate	pentanol
ethyl cinnamate	4-methylbutanol
Acids	hexanol
acetic acid	heptanol
propionic acid	octanol
isobutyric acid	3-octanol
valeric acid	3-octen-3-ol
isocaproic acid	trans-2-octen-1-ol
caproic acid	decanol
heptanoic acid	benzyl alcohol
octanoic acid	1-phenylethanol
trans 2-octenoic acid	2-phenylethanol
nonanoic acid	ethanol
decanoic acid	propanol
phenylacetic acid	

Continued overleaf

Table 2.7(Continued)

Compound
Others
linalool
citronellyl acetate
lavandulole
β-ionone
epozy-β-ionone
benzothiazole
limonene
p-cumene
furfural
acetaldehyde-n-methyl-n-formylhydrazone
pyridine
2-pentylfuran
2-hexylfuran
trans-linalool oxide
cis-linallol oxide
coumarin

Table 2.8 Threshold Values and Odour Characterization of the Main Volatiles in Fresh Mushrooms.

Compound	Threshold Value in ppm	Characterization
1-octen-3-ol	0.010	mushroom-like
1-octen-3-one	0.004	like boiled mushroom, at higher concentrations metallic
trans-2-octen-1-ol	0.040	a little medical, oily, sweet
trans-2-octenal	0.003	sweet, phenolic
3-octanol	0.018	like cod liver oil
3-octanone	0.050	sweet, fruity, musty
octanol	0.48	detergent or soap
1-octen-3-yl acetate	0.09	mushroom-like, soapy
1-octen-3-yl propionate	0.022	rich odour, sweet, fruity mushroom-like
nonanol	0.09	detergent or soap, sweet

Source: Pyysalo(1979)

Table 2.9 The nucleotide Constituents of Edible Mushroom(mg %)

Sample	Extraction Method	Nucleotide Constituents				
		5'-GMP	5'-AMP	5'-IMP	5'-UMP	5'-CMP
dried L. edodes	water extract	156.5	131.6		135.2	114.2
dried L. edodes	perchloric acid extract	24.3	22.9		20.7	
dried L. edodes	0.1N sulfuric acid extract	60.5	34.7	32.1	20.7	
fresh L. edodes	water extract	70.1	54.9		37.6	29.4
fresh A. bisporus	water extract	trace	116.3		65.5	trace

Source: Geng,(1976, 1977)

5'-GMP= 5'-guanylic acid 5'-IMP= 5'-inosinic acid 5'-CMP= 5'-cytidylic acid

5'-AMP= 5'-adenylic acid 5'-UMP= 5'-uridylic acid

Chapter 3

SEPARATION AND IDENTIFICATION OF THE STEROLS IN MUSHROOMS

3.1 INTRODUCTION

The sterols are hydroxylated derivative of the cyclopentanperhydrophenanthrene nucleus. All sterols are capable of forming ester with fatty acids but, in general, the free sterols are more abundant than their ester. Examples of common sterols are: cholesterol, typical of higher animals; β-sitosterol, common in higher plants; ergosterol, typical of gungi(Clark, 1964).

It is easy to extract sterols from the natural material; yet the separation of mixed sterols is difficult. Our research first used the ordinary method to analyse the existence of the sterols of unsaponifiable matter. When this analysis gave positive result, the sterols were isolated with preparative TLC plates and purified by recrystallization. Afterwards, we applied methods of UV, IR and NMR to test the crystal in order to identify which to sterol it belonged. At the same time, we also applied a known samples(authentic samples) to compare their retention times for further confirmation and to observe whether there is contamination by a small amount of other sterols which are hard to remove.

In this chapter emphases have been given to descriptions the isolation and identification of sterols in *V. volvacea* and *T. fuciformis*. In the case of edible mushrooms, only the tests for ergosterol were carried out.

3.2 MATERIAL AND METHODS

3.2.1 Sample collection and pretreatment

From October, 1980 to September, 1981, four crops of *V. volvacea* were cultivated on cotton-waste compost in the Mushroom House specially for this project. After 9-11 days of spawning, some fruiting bodies had reached maturation while others were still in the egg stage. They were harvested them at the same time and separated into two groups: the mature mushrooms and those in the egg stage. Each group was further divided into 3 parts: The whole fungus, cap and the stem(Table 3.1). They was instantaneously frozen in liquid nitrogen and dried by vacuum lyophilization at -60°C. The dried mushrooms retained their natural size and shape, and had a whitish color and native bouquet.

Other edible mushrooms were purchased from the market in Hong Kong and were treated in the same way.

3.2.2 Moisture content

Fresh mushrooms generally contain 85-95% moisture while commercial air-dried ones contain 5-10%. This high moisture content is not unusual, however, if mushrooms are compared with other common vegetables(Crisan and Sands, 1978). The moisture content were determined as follows: About 500 g fresh sample were collected and divided into six parts

as described in 3.2.1. Each was accurately weighed and let stand for air-drying for one day. They were then put into an oven at 45-50°C for 8 hours, 4 hours at 80°C, and finally at 105°C till they reached constant weight. Their moisture content was calculated according to the following formula:

$$\text{Moisture content}(\%) = \frac{A - A'}{W} \times 100$$

where A= fresh weight

A'= dried weight

W= sample weight

3.2.3 Quantitative estimation of lipids

(A) Extraction of lipids(A.O.A.C, 7.044, 1975)

A sample of about 5 g was accurately weighed into a Whatman extraction thimble that has been previously dried at 80°C and cooled in a desiccator. The sample was extracted with about 250 ml anhydrous ether in a Soxhlet apparatus (the flask of which had been dried and weighed) for 24 hours on water bath. Then the solvent was evaporated with a rotary evaporator under reduced pressure. After the solvent had

evaporated, the flask was throughly dried in a vacuum desiccator for 24 hours and weighed. The residue was extracted twice in the same way. Lipid content was calculated as below:

$$\text{Lipid content}(\%) = \frac{W' - W}{DW} \times 100(\text{g}/100\text{g dry sample})$$

where W' = weight of flask + ether extract(g).

W = weight of empty flask(g).

DW= dry sample weight(g).

(B) Extraction of lipids(Beilby, 1981)

About 10g sample was accurately weighed into a separating funnel. 400 ml of chloroform:methanol(2:1) was added and the funnel was shaken for 1 hour. Then 50 ml H_2O was added, mixed well and let stand for a while. The extract(lower layer) was run off into another separation funnel, 25 ml of H_2O was added, shaken well and let stand for a few minutes. The upper layer is the methanol phase and lower layer is the chloroform phase. The methanol layer was discarded, and the chloroform phase was evaporated under reduced pressure. This extraction process was repeated twice more. Lipid content was calculated by the same formula as listed A.

3.2.4 Detection of sterols in intact lipids

The intact lipid mixture can often be separated by TLC and spots of sterols can be visualized on TLC with aid of a variety of spray reagents(Mangold, 1961; stahl, 1969). In this experiment, two chromatoplates were prepared for TLC by coating 20 X 20 cm glass plates with a 0.25 mm layer of a slurry made by mixing 0.6g of $CaSO_4 \cdot \frac{1}{2}H_2O$ and 29.4g of silica gel with 60 ml H_2O by using a specific applicator or placing strips of masking tape on two edges of the glass plates, pouring the slurry in the area between the tape, and then sweeping off the excess slurry with a glass rod resting on both strips of tape. The plates were first air-dried and then oven-dried(110°C,1 hour) before use. A 3-5 μ-litre sample which was prepared in 1% solution of chloroform-methanol(3:1) and the same amount of 1% solution of standard ergosterol(Sigma Co.) were spotted on to the plates in a row 2 cm apart. Then the plates were developed in one of the following two solvent systems: (1) hexane: ethyl acetate(8:2); (2) chloroform:methanol:conc. NH_4OH (75:25:4) for about 30 min.. When the solvent front had moved to the top of the plate, the plate was taken out and air-dried. For visualization, the plate was placed into a chamber containing iodine vapour for half an hour, when yellow spots appeared on the plate. When this plate was taken out from chamber, the yellow spot would vanish quickly

in case of non-ergosterol substances, whereas in case ergosterol the yellow spot would turn to deep green in colour and would not disappear. In a second method of visualization, the plate was sprayed with conc. H_2SO_4 after it was taken out from the iodine vapour chamber. The yellow spot on the plate would change to different colours. The spot of ergosterol would turn green and then light orange.

3.2.5 Quantitative estimation of saponifiable and unsaponifiable matter

About 0.5g of the sample was accurately weighed and put into the saponification flask. Then 10 ml alcoholic solution of 0.5N KOH was added and refluxed on a water bath for an hour. After hydrolyzing, most of the alcohol was evaporated and diluted with water. The mixture was extracted with 6 x 25 ml ether, and the combined ether was washed with water until it showed neutral pH. After drying with anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure. The residue was stored in a vacuum desiccator for 24 hours and weighed. This is the unsaponifiable matter.

After extracting the unsaponifiable matter, the aqueous layer was neutralized with 10% HCl and the resulting fatty acids were extracted with 4 x 25 ml ether. The combined ether was washed with water until it showed neutral pH. After drying with anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure. The residue was stored in a vacuum desiccator for 24 hours and weighed. This is the saponifiable matter. Their ratio were calculated as below:

$$\text{Saponifiable matter}(\%) = \frac{SW}{W} \times 100 (g/100g \text{ lipids})$$

$$\text{Unsaponifiable matter}(\%) = \frac{UW}{W} \times 100 (g/100g \text{ lipids})$$

where SW= saponifiable matter weight

UW= unsaponifiable matter weight

W= lipid weight

3.2.6 Separation of sterols

(A) 0.2g unsaponifiable matter was dissolved in ether and filtered. The filtrates were allowed to stand until most of the ether had evaporated. Then needle crystals appeared, and they were filtered. The mother liquid, after recovery of solvent, was treated twice more in the same way, and crude crystal was obtained. The crude crystal was dissolved in a small amount of chloroform, and methanol was gradually added till turbidity appeared. When this was allowed to stand a flaky crystal separated out.

(B) 0.1g crude crystal was weighed into an acetylation flask, and 10 ml acetic anhydride-pyridine(1:1) was added. This was refluxed for 30 min on a mantle, scale 2-3(Isopad Isomantle, Dorehamwood, Herts, England), cooled to room

temperature and glacial water was added to hydrolyse the excess acetic anhydride, precipitated solid was filtered, washed to neutral pH and then dried in a desiccator. This is its sterol acetate.

The acetate was chromatographed with hexane-benzene (3:2) three times on 20% $AgNO_3$-Si gel TLC plates, and fraction 1 and fraction 2 were obtained(Yokokawa and Mitsuhashi, 1981).

(C) The crude crystal was chromatographed with chloroform-ethyl acetate(7:3) on 10% $AgNO_3$-Si gel TLC plates, and two fractions were obtained.

3.2.7 Identification of sterols

3.2.7.1 Determination of meltion point

The melting point of a small sample of the crystalline fraction 1 was determined by using a small capillary in an liquid paraffin bath. Similarly, the melting point of authentic ergosterol and a mixture of ergosterol and fraction 1 were also determined.

3.2.7.2 Liebermann-Burchard test

Three test tubes were prepared , the first containing 2 ml of chloroform; the second, 3-5 mg of fraction 1 in 2 ml of chloroform; and the third, 3-5 mg of ergosterol in 2 ml of chloroform. 1 ml of acetic anhydride was added to each and mixed well. Then added 2 ml of concentrated H_2SO_4

to each, and mixed cautiously. The second and third test tubes yielded a positive Liebermann-Burchard reaction.

3.2.7.3 Thin layer chromatography

Identical with 3.2.4 and 3.2.5.1.

3.2.7.4 Gas liquid chromatography

The sterol fraction from V. volvacea obtained by preparative TLC was further fractionated after acetylation. The acetates were analysed by GLC. The instrument used was a Hewlett-Packard 7620A Research Chromatograph. The glass column(2 m X 3 mm i.d.) was packed with 3% OV-17 chromosorb W AW, flow rate of helium the carrier gas, was 50 ml/min. Oven, detector and injector temperature were 240°C, 245°C, 245°C repectively. Sterols were identified by comparison of their relative retention times with those authentic samples.

3.2.7.5 Spectrographic methods

UV spectra were measured in ethanol(1 %). IR spectra were recorded in KBr pellets. NMR spectra were measured on a 100 MHz instrument in $CDCl_3$ with TMS as internal reference.

3.2.8 Quantitative estimation of ergosterol and other sterols

About 0.5g of unsaponifiable matter was weighed

accurately. It was acetylated by the acetic anhydrieds-pyridine. The acetylated substance was transferred to a 10 ml volumetric flask. Exactly 10 ml of chloroform was added, and 0.1 ml aliquots were spotted on 8 pieces of TLC plates (5 cm X 20 cm) with a graduated micro-pipette. The plates were developed solvent of hexane:ethyl acetate(9:1). When the solvent reached the top, the plate was taken out and the solvent allowed to evaporate. The position of the sterol acetate was fixed under illumination with UV light. The spot was extracted by chloroform and moved to a 5 ml volumetric flask. Exactly 5 ml of chloroform was then added, 0.005 ml of the solution was applied to GLC analysis, using cholesterol acetate as internal standard. By the relation between the peak area and weight, the amount of ergosterol in the unsaponifiable substance could be calculated. The content of other sterol was determined by ratio of sterol fraction to the total peak area. The calculation of the ergosterol content is as follows:

Ergosterol content(% of unsaponifiable matter)=

$$\frac{EA \times IW \times D}{IA \times UW}$$

where EA= area of ergosterol
IA= area of internal standard
IW= weight of internal standard
UW= weight of unsaponifiable matter
D= dilution factor

Table 3.1 Source of Experimental Materials

Sample	Source	Stage	Part
*Volvariella volvacea**		egg	whole
		egg	cap
		egg	stem
		matured	whole
		matured	cap
		matured	stem
Lentinus edodes			
Dongko (standard grade)	China	matured	whole
Hongshin (Koshin, Hyangshin)	China	matured	whole
Kwangtung Hongko (north mushroom)	China	matured	whole
Cracky Dongko (best grade)	Japan	matured	whole
Agaricus bisporus	Japan	egg	whole
*Pleurotus sajor-caju**		matured	whole
Auricularia auricula	China	matured	whole
Tremella fuciformis	China	matured	whole

Note: * Experimental Mushroom House of Biology Department

3.3 RESULTS

3.3.1 Total lipid content and sterols

The yield of total lipid content in caps of *V. volvacea* of different stages, with three pretreatments and two extraction methods is shown in Table 3.2. It is to be noted that the fresh material, as well as the material lyophilized after harvesting, gave a much higher yield than that predried in the oven. The caps of matured stage, for example, gave 5.4% and 5.9% yield of lipid respectively in using lyophilization and fresh material but gave 3.1% only in oven-dried material. There is about 43% loss of lipid in oven-dried pretreatment material when it is compared with other two treatments. However, it seems there is no significant different in lipid content between the fresh and lyophilized materials. A negligible difference between two extraction methods was observed.

The TLC results of the intact lipid shown in Figures 3.1 and 3.2 indicate that all six edible mushrooms analysed in this study contain sterols. It has been known that the structures of many sterols are very similar. Therefore, a single spot in the silica gel TLC plate may represent a group of compounds. When the 10% $AgNO_3$-Si gel plate was used, some of components then can be separated as demontrated correspondingly in Figures 3.3 and 3.4.

3.3.2 Sterols of V. volvacea

The lipid of V. volvacea was hydrolized with alkali to produce the unsaponifiable matter. This was then recrystallized several times in solvent of MeOH-Me_2CO(1:1). A colourless crystal was obtained. This crystal is named as V1 which showed a positive reaction in Leibermann-Burchard test. The melting point of this crystal is 158-160°C, and the mixed melting point was not depressed by authentic ergosterol(Sigma Co.).

The Rf of V1 and authentic ergosterol in TLC is identical (0.32). It gave a yellow spot when treated with iodine vapour and blue spot with concentrated sulphuric acid. The retention times of GLC analysis of V1 and authentic ergosterol were 22 min.; that of cholesterol was 17 min and ergost-7-en-3β-ol 28 min.

The UV spectra of V1 and authentic ergosterol are the same(Fig. 3.5): (λ nm) 260, 271, 281.5, 293.5. The UV spectrum showed a typical Δ 5,7-sterol absorption. The IR spectrum of V1 and authentic ergosterol are also the same (Fig. 3.6): it shows absorption for a hydroxy(3370 CM^{-1}) and double bonds(1650, 1620 CM^{-1}). ^{1}HNMR spectrum of V1 and authentic ergosterol(Fig. 3.7 and 3.8) showed a characteristic of multible methyl peak of sterol. It had signals with values of 0.61(3H, C-18), 1.04(3H,C-19), 1.15(3H, C-21), 0.90(3H, C-26),0.92(3H, C-27), 1.05(3H, C-28).

The MS revealed a molecular ion at m/e 396(M^+).

As a conclusion, the crystal V1 obtained from the extracts of V. volvacea was confirmed to be ergosterol.

The mother liquid of unsaponifiable matter of V. volvacea after isolation of V1, was evaporated to remove the solvent. Acetic anhydride-pyridine(1:1) was added and it was refluxed to prepare steryl acetate. The acetate was chromatographed with hexane:ethyl acetate(9:1) on preparative TLC plates, and a sterol fraction was obtained. This was then read for GLC analysis. Sterols were identified by comparison of their relative retention times with authentic samples. There are three peaks in the sterol fraction(Fig. 3.9): peak 1 unknown, 2 ergosterol(Rt 22 min.) and peak 3 ergost-7-en-3β-ol(Rt 28 min.) (Table 3.3).

3.3.3 Sterols of T. fuciformis

A crude crystal was obtained in recrystallization of the unsaponifiable matter in MeOH-Me_2CO. The crude crystal was chromatographed with chloroform-ethyl acetate (7:3) on 10% $AgNO_3$-Si gel TLC plates and two fractions were obtained(Fig. 3.3). Fraction 1(Rf 0.72) was recrystallized with MeOH-Me_2CO and a colourless needle crystal(T1) obtained. T1 gave positive reaction in Leibermann-Burchard test. The melting point was 146-147°C.

A clear absorption pattern was not obtained in the UV

spectrum of T1, which indicated that a conjugated double bond was not present. The IR spectrum of T1 shown in Figure 3.10, it showed a absorption for a hydroxy at 3448 CM^{-1} and for a double bond at 1644 CM^{-1}. The MS spectrum(Fig. 3.11) showed a molecular ion at m/e 400, as well as fragment ions at m/e 385, 340, 325, 273, 255 and 175. The peak at m/e 255 was characteristic of the fragment ions in sterols with a $\Delta 7$-unsaturated structure. All the above showed that T1 showed be ergost-7-en-3β -ol. The fraction 2(Rf 0.32) was identified as ergosterol on the basis of the GLC results, melting point and the IR spectrum. It is concluded that the sterols of T. fuciformis consist of ergost-7-en-3β -ol and ergosterol.

3.3.4 Sterols of other specimens

In this section, our research focuses only on whether ergosterol is present in other edible mushrooms. The results of the GLC analysis of sterols shown in Table 3.3 demonstrated that ergosterol was present in all six tested mushrooms.

3.3.5 Quantitative estimation of ergosterol and other sterols

The result of ergosterol content in the six edible mushrooms(Table 3.4) has shown that the ergosterol content of V. volvacea is the highest, the second is L. edodes and

A. bisporus, and T. fuciformis ergosterol is the lowest. But T. fuciformis contains 93% of ergost-7-en-3β-ol its in sterol fraction. V. volvacea contains 40.1% of ergost-7-en-3β-ol(Table 3.3). Besides ergosterol, there is a considerable amount of unidentified sterol in the sterol fraction of A. auricula. These are the common properties of the sterol composition in the above described edible mushrooms.

The ergosterol content is higher in the matured stage than the egg in V. volvacea. In these two different stages, the cap contains more ergosterol than the stem(Table 3.5). The difference lies in the total lipid content. That is to say, the matured cap contains more lipid. If we just compare the unsaponifiable matter, the reverse will be true, i.e., the stem of the egg stage contains more ergosterol. This finding is similar to that of Yoshicha, et al.(1979) on L. edodes.

Table 3.2 Total Lipid Content of *V. volvacea* with Different Pretreatments and Different Extraction Methods

Stage	Part	Pretreatment	Extraction Method	Yield(%)
egg	cap	I	*	3.9
egg	cap	II	*	2.9
matured	cap	I	*	5.4
matured	cap	II	*	3.1
matured	cap	III	**	5.9
matured	cap	I	**	5.5
matured	cap	II	**	3.1

Note: I lyophilization dried, II oven dried, III fresh

* see 3.2.3A, ** see 3.2.3B.

Table 3.3 Compositions of Sterol Fractions(%)

	I	II	III	IV
Volvariella volvacea	54.1	40.1		5.8
Lentinus edodes				
Dongko (standard grade)	79.2		30.8	
Hongshin (Koshin, Hyangshin)	69.2		30.2	
Kwangtung Hongko (north mushroom)	52.4		47.6	
Cracky Dongko (best grade)	66.6		33.4	
Agaricus bisporus	56.1			43.9
Pleurotus sajor-caju	54.7			45.3
Auricularia auricula	46.1			53.9
Tremella fuciformis	6.8	93.2		

Note: I ergosterol, II ergost-7-en-3β -ol
III ergosta-5,7-dien-3β -ol, IV unknown

Table 3.4 Total Ergosterol Contents of *V. volvacea* and Other edible Species of Fungi

	Total lidip (% of dry material)	Unsaponifiable materials (% of ether extract)	Ergosterol (% of unsapon.)	Ergosterol (% of dry material)
Volvariella volvacea	3.0	32.4	48.4	0.47
Lentinus edodes				
Dongko (standard grade)	2.1	21.8	46.8	0.21
Hongshin (Koshin, Hyangshin)	2.0	25.8	30.2	0.15
Kwangtung Hongko (north mushroom)	1.3	18.3	23.9	0.06
Cracky Dongko (best grade)	2.1	28.0	47.6	0.27
Agaricus bisporus	3.1	21.7	33.6	0.23
Pleurotus sajor-caju	1.6	25.0	35.5	0.13
Auricularia auricula	1.3	25.1	22.8	0.07
Tremella fuciformis	0.6	19.5	7.4	0.01

Table 3.5 Total Ergosterol Contents of Different Stages and Different Parts of *V. volvacea*

Stage	Part	Total lipid (% of dry material)	Unsaponifiable material (% of ether extract)	Ergosterol (% of dry material)
matured	whole	3.6	30.6	0.54
matured	cap	5.3	25.0	0.63
matured	stem	1.4	40.5	0.27
egg	whole	2.6	31.1	0.39
egg	cap	3.5	32.3	0.53
egg	stem	1.5	35.3	0.25

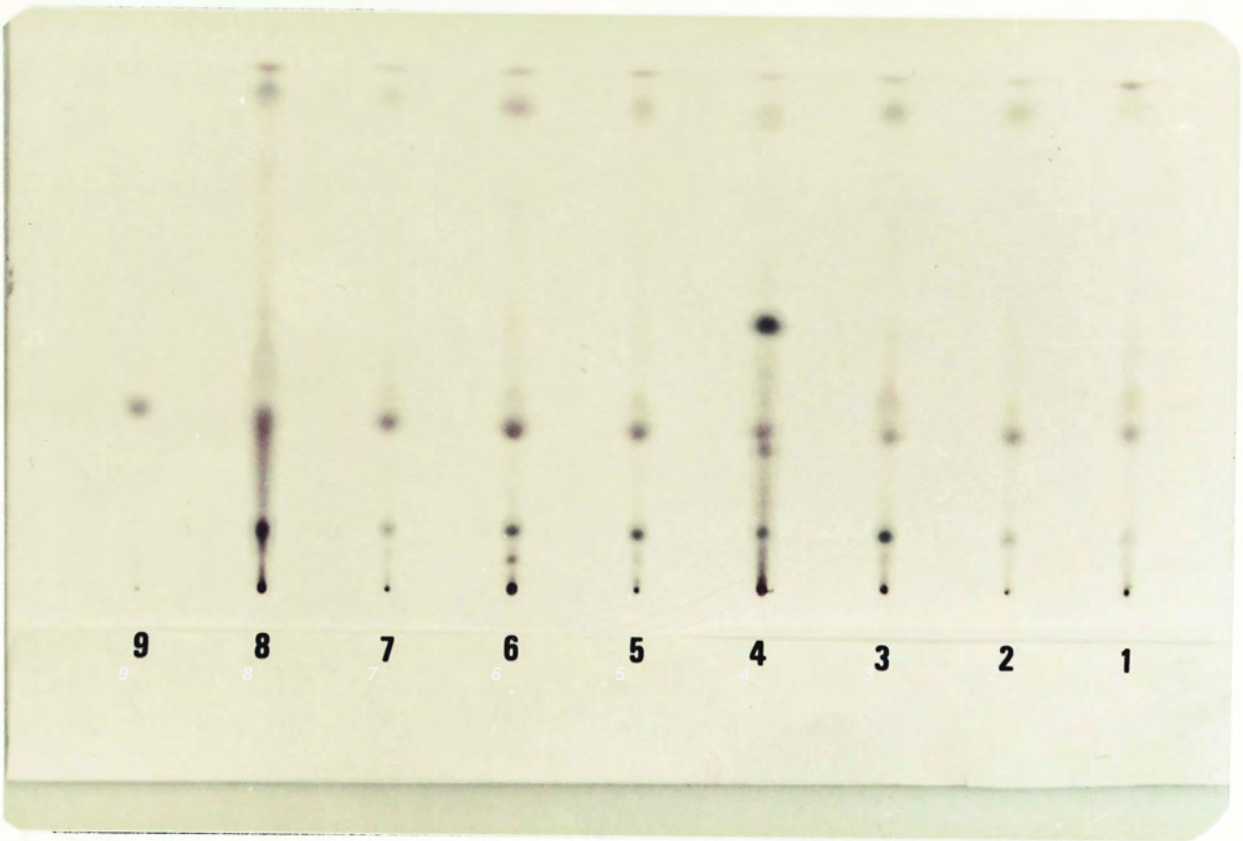


Fig. 3.1 The TLC Chromatogram of Intact Lipid of Edible Mushroom on Silica Gel

1, T. fuciformis
2, A. auricula
3, L. edodes(Hongshin)
4, A. bisporus
5, L. edodes(Kawangtung Hongko)
6, L. edodes(Cracky Dongko, Japan)
7, L. edodes(Dongko)
8, V. volvacea
9, authentic ergosterol

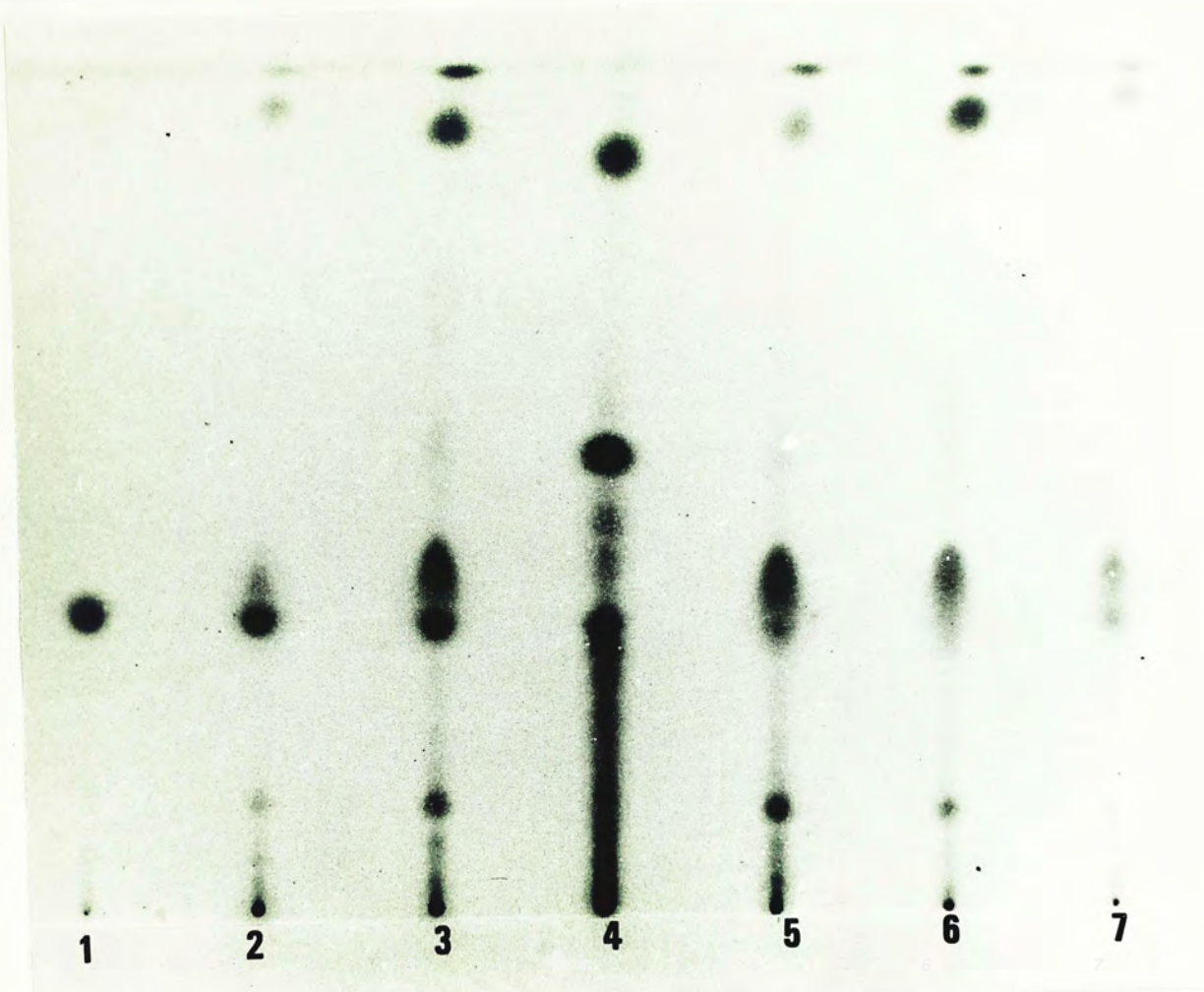


Fig. 3.2 The TLC Chromatogram of Intact Lipid of Edible Mushrooms on Silica Gel

1, authentic ergosterol
2, V. volvacea
3, L. edodes
4, A. bisporus
5, P. sajor-caju
6, A. auricula
7, P. fuciformis

Fig. 3.3 The TLC of Sterols on Silica Gel

1, authentic ergosterol

2, The sterol fraction of V. volvacea

3, The sterol fraction of T. fuciformis

Fig. 3.4 The TLC of Sterol on 10% $AgNO_3$-Si gel

1, authentic ergosterol

2, The sterol fraction of V. volvacea

3, The sterol fraction of T. fuciformis

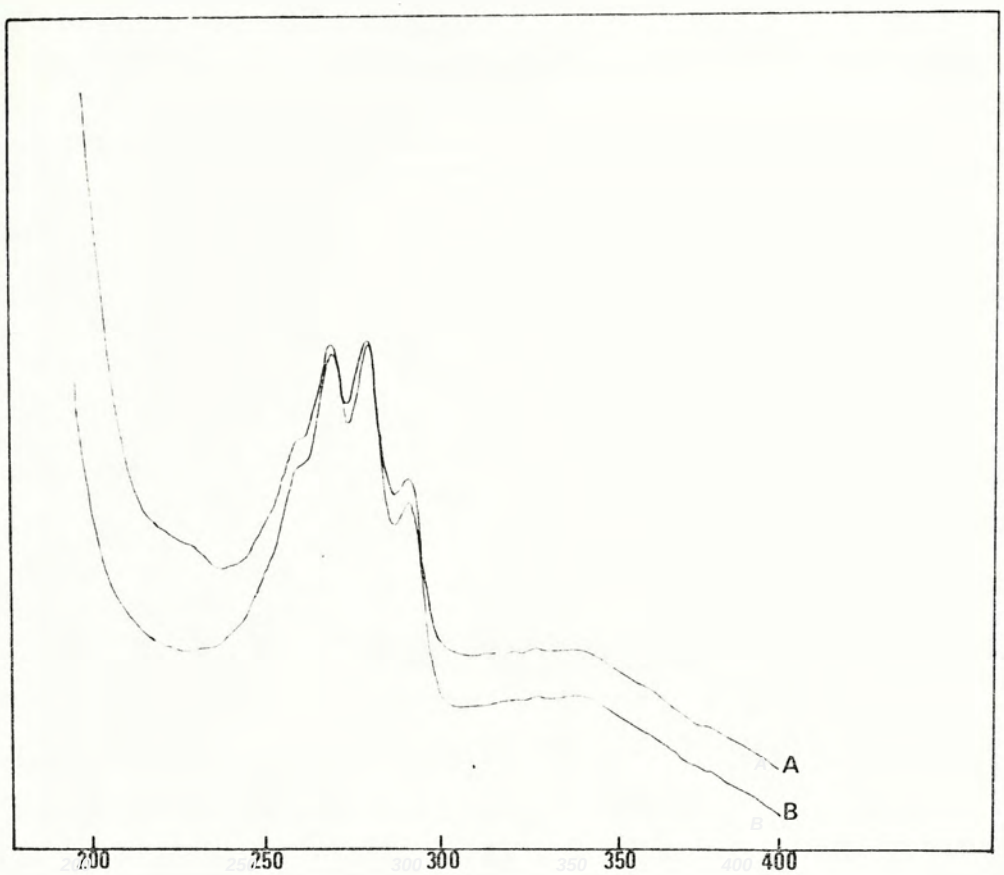


Fig. 3.5 The UV spectra of V1 and authentic ergosterol

A: authentic ergosterol

B: V1 of *V. volvacea*

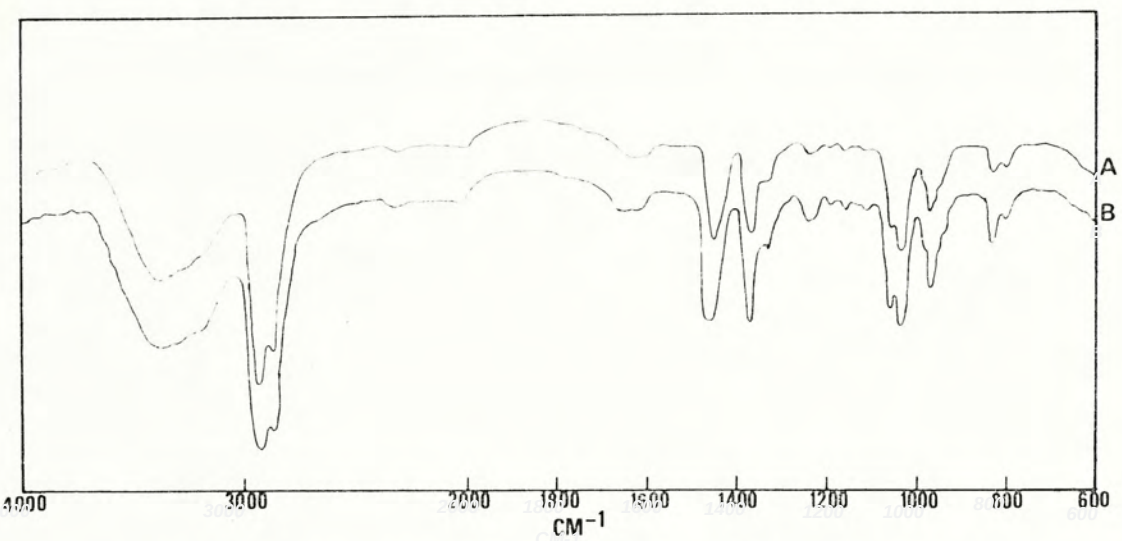


Fig. 3.6 The IR spectra of V1 and authentic ergosterol

A: authentic ergosterol

B: V1 of V. volvacea

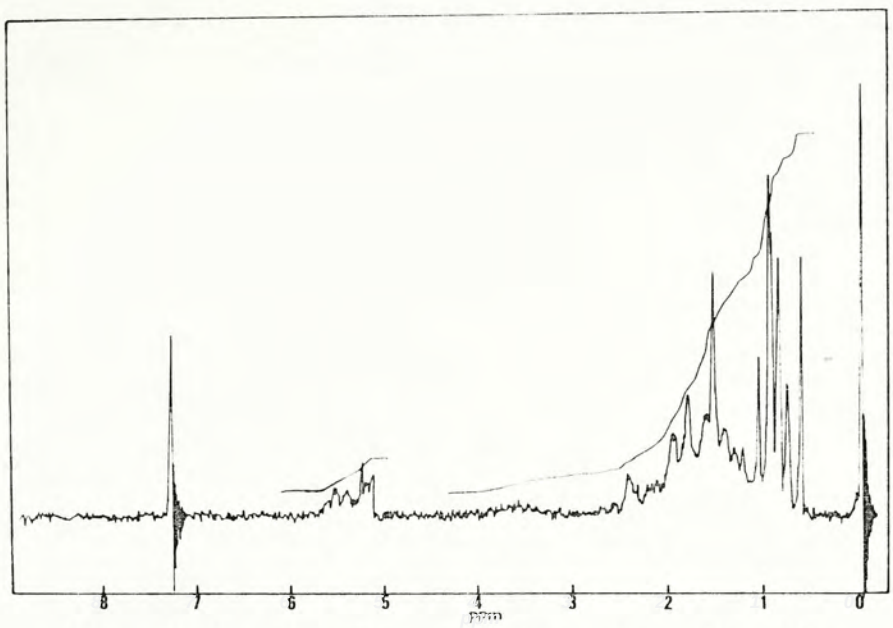


Fig. 3.7 The NMR spectra of authentic ergosterol

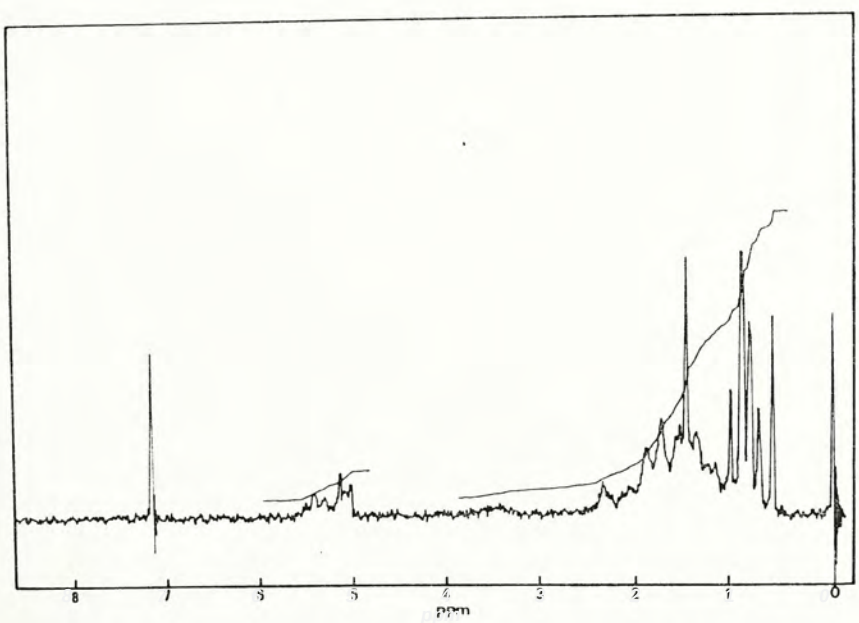


Fig. 3.8 The NMR spectra of V1 of V. Volvacea

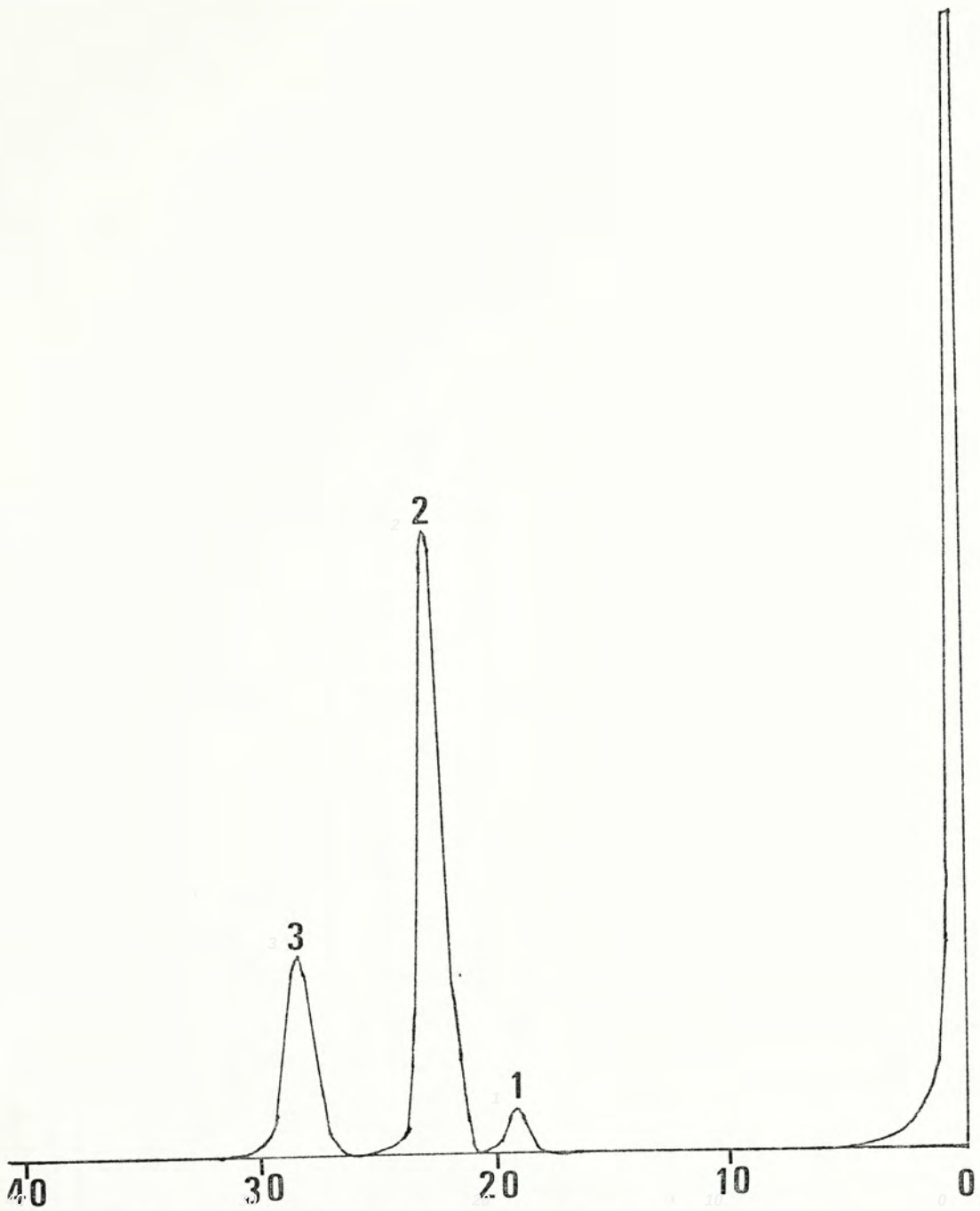


Fig. 3.9 Gas chromatogram of sterol fraction in V. volvacea

peak 1: unknown
2: ergosterol
3: ergost-7-en-3β-ol

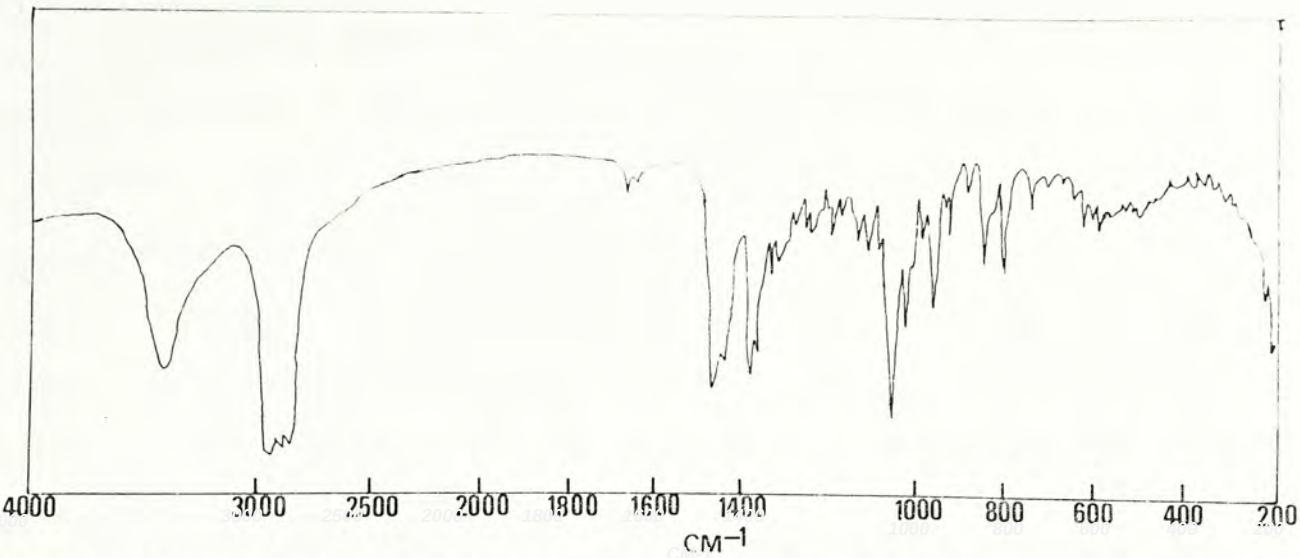


Fig. 3.10 The IR spectra of T1 of T. fuciformis

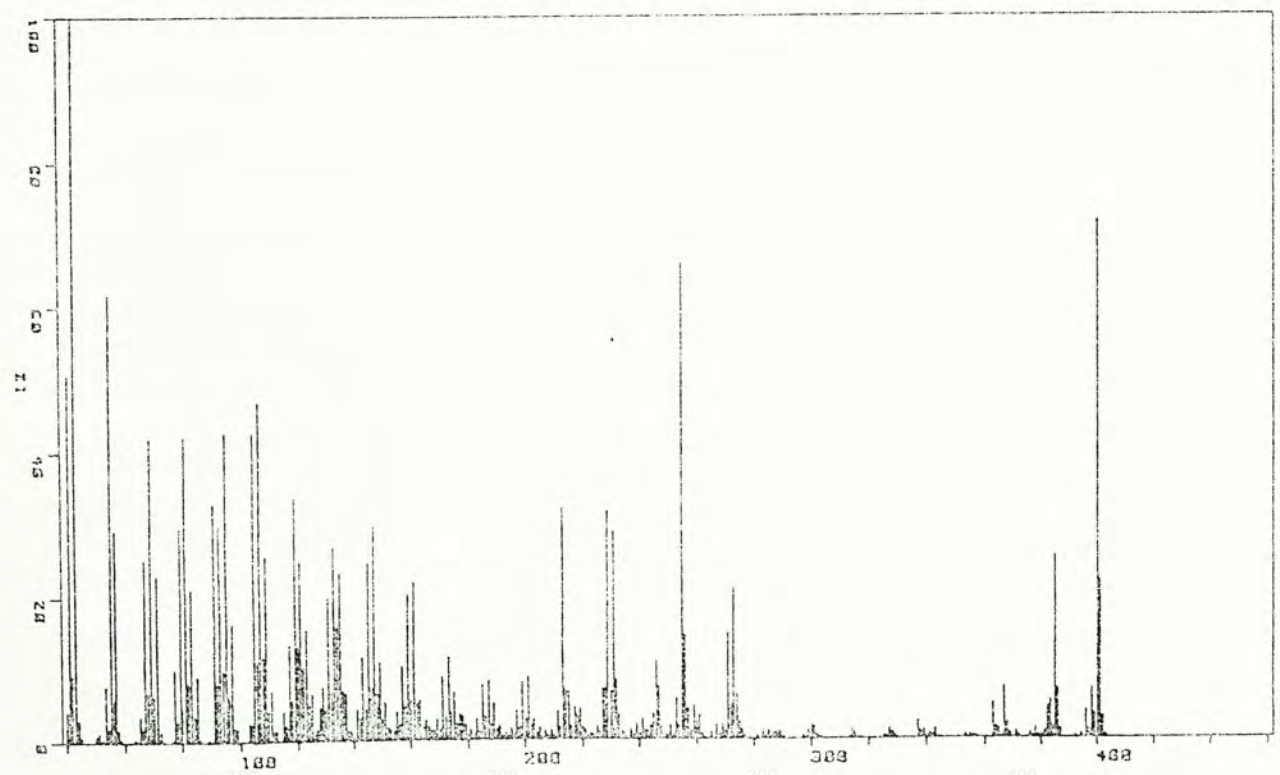


Fig. 3.11 The MS spectra of T1 of T. fuciformis

3.4 DISCUSSION

Since fresh mushrooms have a high water content(Table 3.6), there are difficulties in the pretreatment of the materials. For an investigation of the chemical constituents of the edible mushrooms, the best way of drying is by lyophilization(mentioned in 3.2.1). After harvest, the fresh mushroom should be immediately frozen by liquid nitrogen: this is to avoid further action of enzymes to decompose the chemical constituents. In addition, this can avoid the decomposition caused by high temperature when drying method is used.

It is particularly worth notice here that many mushrooms contain high levels of peptides and protein; these form hard pieces in heating so that the materials are not only hard to disruption, but the organic solvent is also prevented from diffusing into the material during extraction. Using two different methods of treatment on V. volvacea proves this analysis(Table 3.2).

The extraction of lipid is by either reflux in ether or shaking extraction in chloroform:methanol(1:1 or 2:1) below room temperature(Burton, 1974). The latter method provides a means to separate the water-soluble substances and fat-soluble lipid and also the yield is higher but the non-lipid content increased too. Our experiment focuses on the fat-soluble lipid, and we therefore adopted the ether

extraction; the material being treated with ether for 5 to 8 hours(A.O.A.C, 1978). We have comprared the time effect and the lipid yield, and have found that in the first 8 hours, the yield is 2.3%, the next 8 hours(totally 16 hours), 2.84%, the third 8 hours(totally 24 hours) 3.05%. Therefore, ether is used and the mixture is heated in reflux for 24 hours. The yield is comparable to that obtained from the method of chloroform-methanol extraction yet the non-lipid substance is much reduced in our extraction. This would be advantageous to the further separation.

There are a variety of sterols in the natural product. The sterols in fungi have been reviewed by Weete and Weber (1980). They supported the conclusion that there is wide distribution of ergosterol in fungi with yeast in having the greatest amount but the widest distribution in the Basidiomycetes. Also, they pointed out that there are other sterols besides ergosterol. Yokokawa and Mitsubashi (1981) in analysing 10 species of Basidiomycetes proved the existence of ergosterol(Table 3.7). Our experiment analysed 6 species of Basidiomycetes and all contained ergosterol (Table 3.3). From the above, we conclude that ergosterol is a representative sterol in the Basidiomycetes.

Fungi contain various kinds of sterols which can now be isolated readily. The best and the quickest method is the application of GC-MS(Lisboa, 1974; Perkins, et al.,

1968) which, unfortunately, was unavailable to us. Nevertheless, we integrated the applications of various new techniques, e.g. the isolation and identification of ergosterol and ergost-7-en-3β-ol in V. volvacea and T. fuciformis were applied by preparative $AgNO_3$-Si gel TLC plates. The identification of crystal obtained is according to the RR_t on GLC and further tests were carried out by UV, IR, MS and NMR, these ensuring the accuracy of the identification. Hence, we have reached the GC-MS standard in identification of sterols.

We have already pointed out that mushroom must be treated in a uniform way if valid comparisons of their ergosterol contents are to be made. For example, L. edodes contains abundant ergosterol, yet the reported data of various researchers differs greatly. Yoshica et al.(1979) reported the L. edodes contained 0.22% of ergosterol but Yokokawa and Mitsuhaki(1981) reported that it contained 0.51%. Our result is close to that of Yoshica et al.

The roles of sterols are two fold: one is in organism itself; the other is in the human. "Although Schönheimer, et al.(1930) stated that phytosterols are waste products, Hoftmann(1971) reviewed the function of phytosterols and aggested that they probably have the same function as in animal i.e., they act as membrane components, as hormones and as steroid precursors"(Bean, 1973).

Research on the sterol's action on human beings is much limited. It is well known that ergosterol is the precursor of vitamin D which could be formed by irradiation with an artificial light source of a fluorescent sunlamp. In all six tested mushrooms, they contain ergosteral such that the belief that mushrooms have nutritive and medical effects is further strengthened by this study.

Table 3.6 Moisture Content of Different Stages and Different Parts of Volvariella volvacea

Stage	Part	Moisture(%)
egg	whole	88.6
egg	cap	88.1
egg	stem	89.1
matured	whole	88.8
matured	cap	91.2
matured	stem	86.5

Table 3.7 Compositions of Sterol Fractions(%)

	I	II	III	IV	V	VI
Hygrocybe punicea			78.8		21.2	
Lampteromyces japonicus			45.6		54.4	
Leucopaxillus giganteus	6.8		85.9		7.3	
Lentinus edodes			83.6		16.4	
Flammulina velutipes		30.1	45.3	14.1	10.5	
Amanita caesarea			82.5		17.5	
Coprinus atramentarius			24.5		75.5	
Russula foetens			70.9		29.1	
Russula nigricans			83.8		16.2	
Russula senecis			84.5			15.5

Source: Yokokawa and Mitsugashi(1981)

I: cholest-7-en-3β -ol, II: ergosta-5,8,22-trien-3β -ol
III: ergosterol, IV: ergosta-7,22-dien-3β -ol
V: ergosta-5,7-dien-3β -ol, VI: ergost-7-en-3β -ol.

Chapter 4

SEPARATION AND IDENTIFICATION OF FATTY ACIDS OF *VOLVARIELLA VOLVACEA* AND OTHER EDIBLE SPECIES OF FUNGI

4.1 INTRODUCTION

A brief review of the trends in fungal lipid research is revealing. In 1870's it was reported that Ergot fungus, *Claviceps purpurea*, contained 30% fat. During the following 50 years most work of this nature was devoted to screening fungi for potential high fat producers and it was recognized that fungi vary considerably in their capacity to produce fat. Perhaps more importantly, however, it was recognized that the degree of fat production varies according to the culture condition and media composition. Research in the following half century emphasized the importance of defining cultural conditions and nutritional factors which are favorable for fat production (Waete, 1980).

Nowadays, research an edible mushrooms, of course, no longer treats fat production as the main theme. Yet, research on fats has never stopped, and has been stimulated by the application of GLC which gives better results in the identification and quantitative analysis of fatty acids in mushrooms. It is a pity that the analysis of the fatty acids content on several main edible mushrooms were worked out by different groups of researchers so that the reported data varied greatly.

This not only makes it difficult to compare the fatty acid content among them, but also cannot provide very precise results for the fat content of even one among them. With regard to these, our research was purposed to analyse the fatty acid content of several edible mushrooms under uniform conditions. Therefore, our data could give a better position to compare and evaluate the fatty acid content of these tested mushrooms. Besides, we have analysed the fatty acid content of different stages and different parts of V. volvacea. This was to provide a data basis for the appropriate timing of harvest.

4.2 MATERIAL AND METHODS

4.2.1 Sample collection and pretreatment

see section 3.2.1

4.2.2 Preparation and isolation of fatty acids

Prior to separation and identification, it is convenient to obtain natural fatty acids either as free fatty acids or their simple alkyl esters. The free fatty acids are converted into a variety of derivatives which are necessary for identification. This usually involves saponification (see 3.2.6.1), purification of fatty acids, and preparation of derivatives.

4.2.2.1 Purification of fatty acids(Kuksis, 1978)

For the purpose of GLC, sufficient fatty acids are purified by TLC using a neutral solvent system which contains acetic acid to ensure that all the fatty acids are in an ionized form during chromatography. Two systems that give good results (since they separate free fatty acids of various chain length from esteryl ester, glycerols, free sterols etc.) are hexane: diethyl ether:acetic acid(85:15:2) and heptane:isopropyl ether: acetic acid(60:40:4). The free fatty acids are recovered from TLC plates by extraction with chloroform:methanol(2:1). The extract is evaporated on a water bath under reduced pressure and then dried.

4.2.2.2 Preparation of derivatives

Although free fatty acids of short chain length(C_2-C_6) may be effectively employed for both chromatographic separation and instrumental identification, the longer chain acids are best handled in the form of a simple alkyl ester. The fatty acids of each lipid calss usually have been prepared in the form of fatty acid methyl esters. The method of preparing fatty acid methyl ester is as follows(Wijngarrden, 1976):

About 0.5 g of free fatty acids were placed into a round bottomed flask and 10 ml 14% BF_3(Boron trifluoride-methanol) in methanol was added. After reflux for 30 minutes, 20 ml hexane was added to the mixture through the condenser and boiled for 10 minutes. The mixture was allowed to cool and saturated NaCl solution was added. The mixture was transferred into a separating funnel. The upper hexane phase containing methyl ester of fatty acids was separated from the lower aqueous phase. The aqueous phase was extracted three times with each 30 ml hexane. The four extracts were combined and washed with water until neutral in pH. The solvent was evaporated by rotary evaporator under reduced pressure and the methyl ester of fatty acids were obtained.

4.2.2.3 Purification of fatty acid ester

In addition to chain length and unsaturation homologues, the fatty acid ester mixture may contain keto, hydroxy,

cyclopropane, and epoxy derivatives, as well as dimethylacetals. The sample was purified by chromatography on a small silica acid column. The fatty acid methyl esters were recovered by elution with 1% diethyl ester in hexane. The other substances remained on the column.

4.2.3 Identification of fatty acid methyl ester

4.2.3.1 Separation of saturated and unsaturated fatty acids methyl ester(Cook and An, 1975).

Synthesis of $Hg(Ac)_2$ adduct: A ample of 150mg of methyl ester of fatty acids and a portion of 750mg of mercuric acetate were placed in a flask and 50 ml of a solution containing 5% distilled water and 0.3% glacial acetic acid in methanol was added. The mixture was heated on a water bath at 60°C for approximately 10 min.. To ensure solution of the mercuric acetate, the flask was stored in the dark at room temperature for 24 hours. The solvent and excess acetic acid were removed and the residue was dried by evaporation at room temperature. The dry residue was shaken several times with 50 ml benzene at 50-60°C, and the extracts were filtered through glass wool into a column of silica gel.

Column chromatography: The silica gel column was prepared from a slurry in benzene which was poured into glass column (50 cm in height). The column was eluted with the benzene to a total volume of 500 ml. This eluate contained the methyl

esters of saturated fatty acid, together with unsaturated fatty acid mercuric acetate adducts remaining at the top of the column as indicated by a yellow band.

The mercuric acetate adducts were eluted with 150 ml of 5% glacial acetic acid in absolute ethanol. To recover the methyl ester of the unsaturated fatty acids, this eluate was treated with 50 ml of 6N hydrochloric acid and 300 ml of water. After 10 min. this mixture was diluted with 500 ml water and extracted with 100 ml of benzene. The resulting benzene fractions were dried with anhydrous sodium sulfate and evaporated to dryness under vacuum on a rotary evaporator. The methyl ester of unsaturated fatty acid were obtained, and then used for TLC or GLC analysis.

4.2.3.2 Identification of unsaturated fatty acids by TLC

The fatty acids of edible mushrooms are mainly unsaturated. $AgNO_3$-Si gel TLC can be used to analyze unsaturated fatty acids (Mangold, 1968), therefore, silica gel conatining 5-10% silver nitrate was almost exclusively used. The procedures or preparation of the slurry and plates have been given in 3.2.7.3. The 10% $AgNO_3$-Si gel plates were air dried like ordinary layers and then activated by heating for 30 min. at 110°C. The mixed fatty acid methyl ester were chromatogramned with hexane: diethyl ether(9:1) on plates with authentic fatty acids methyl ester as a control. All the fatty acids methyl ester

can be visualised by spraying with aqueous sulphuric acid (Mangold, 1969).

4.2.3.3 Identification of fatty acids by GLC

An effective identification of fatty acids by molecular weight or carbon number can be obtained on a nonpolar column by GLC. The instrument used for this analysis was a Hewlett Packard 7620A Research Chromatograph and the measurement conditions are shown in Table 4.1.

The semilog plot of the log of the retention time versus carbon atoms in acid is shown in Figure 4.1 and this fact was established for the identification of various components of fatty acid described by Littlewood(1970) and Ruseva-Atanasona (1966). The above method was used to identify fatty acids of the edible mushrooms in our experiments.

4.2.4 Quantitative estimation of fatty acids by GLC

Two types of quantitative estimation of fatty acids have been commonly performed by GLC. One provides the percent composition of the fatty acid mixture which may be expressed on a mole or weight basis.

The other quantitative analysis involves estimation of the fatty acid content of a lipid fraction. This is best made by means of dilution with a suitable internal standard, such as pentadecanoic, heptadecanoic, and eicosanoic acids, which

Usually occur in natural fatty acid mixture only in trace amounts. For this purpose, the internal standard is added in a ratio of 10~30% depending of the complexity of the mixture of the fatty acid. The method of quantification of fatty acids by means of an internal standard is most useful and very simple. So this method was used to estimate fatty acid content in our experiment.

About 0.5g saponifiable matter was accurately weighed and then esterified as described in 4.2.2.2. The fatty acids methyl ester was transterred into a 10 ml flask, and a definite amount of pentadecanoic acid methyl ester was added as an internal standard. The fatty acid content is calculated by the comparison of their area and the area of the internal standard. The percentage can be represented as follows:

$$\text{Percentage of fatty acid} = \frac{SA \times IW \times D}{IA \times SW}$$

where SA: area of fatty acid in mixed sample

IA: area of internal standard

IW: weight of internal standard

SW: weight of sample

D: dilute factors

Table 4.1 Measurement Conditions(GLC)

Column	6% DEGS, on 80-100 mesh diatoport S 3mm x 2m stainless steel	
Detector	flame ionization detector	
Temperature	injection port 180°C, column 170°C, detector 180°C	
Flow rate	He	60 ml/min.
	H_2	50 ml/min.
	Air	40 ml/min.
Attenuation	4×10^3	

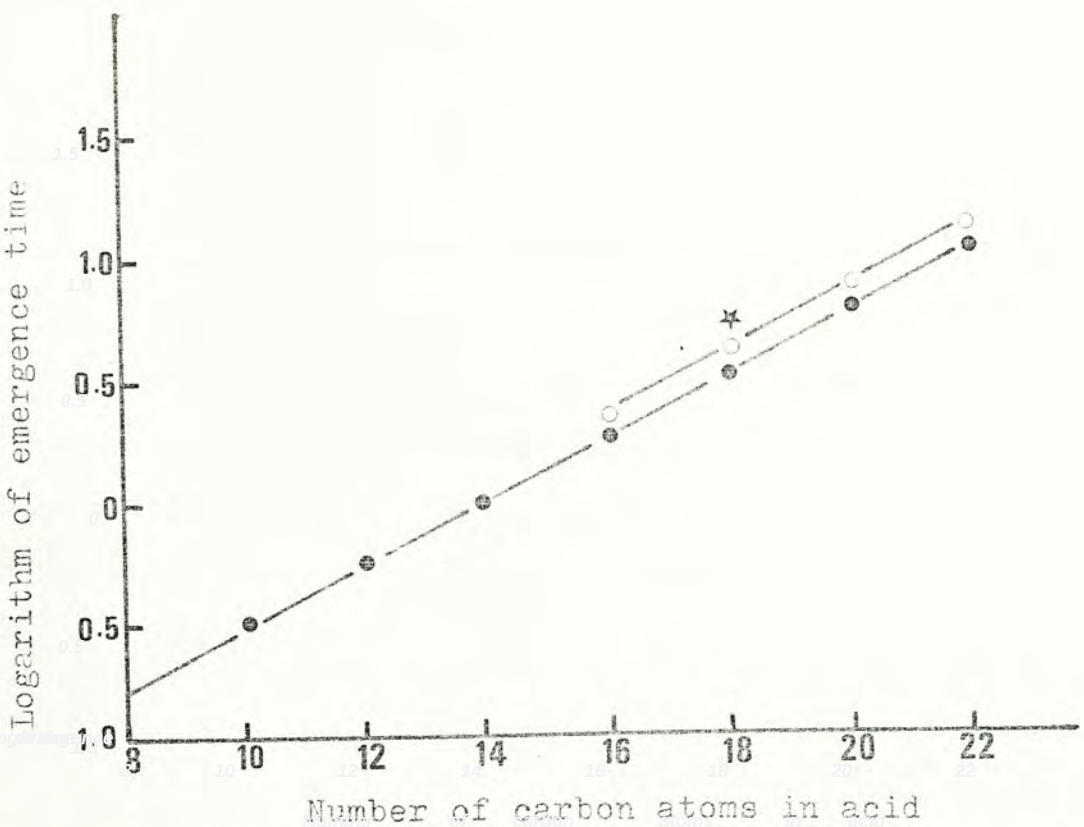


Fig. 4.1 Plot of the logarithms of the relative retention of fatty acid ester against carbon number

● straight chain saturated acids

○ mono-unsaturated acids

✶ di-unsaturated acids

4.3 RESULTS

As shown in Table 4.2, the lipid content of the edible mushrooms ranges from 0.6 to 3.1%. The highest one is A. bisporus, 3.1%, the second is V. volvacea, 3.0%, and the lowest is T. fuciformis, 0.6%. Conversely, the content of saponifiable matter in the lipids is highest in T. fuciformis at 78.1%, while that of V. volvacea is lowest at 58.5%. The reason for this is that the lipids of the latter contains large amounts of ergosterol so that the saponifiable matter is comparatively lower.

Applying GLC (Figs. 4.2 and 4.3) and TLC (Fig. 4.4) on the fatty acids analysis of V. volvacea and the other five edible mushrooms shows that all have a high percentage of unsaturated fatty acids (Tables 4.2 and 4.3). Among them, V. volvacea has reached as high as 83.3%, whereas that of L. edodes is 75.9%; A. bisporus 74.1%; P. sajor-caju 76.6%; A. auricula 73.1% and T. fuciformis 69.2%. This clearly illustrates that the unsaturated fatty acids constitute an important and unique characteristic of the edible mushrooms.

In regard to the individual fatty acids the results shown in Table 4.3 indicated that these six mushrooms contain myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid. It is also noted that oleic acid is the chief fatty acid in T. fuciformis; and linoleic acid is only chief fatty acid in the other five edible mushrooms.

The conten of linoleic acid in these edible mushrooms ranges from 27.98 to 76.25%. Among them, L. edodes has the highest content of 76.25%; the next is V. volvacea 69.91% and the lowest content is T. fuciformis 27.98%.

From figs. 4.2 and 4.3, we can see that V. volvacea contains mainly the six even-number carbon chain fatty acids as mentioned in above. However there are some small fractions of other unidentified fatty acids, which may be the odd-carbon chain fatty acids according to the analysis of the RR_t of GLC after the separation of saturated and unsaturated fatty acids by the chemical method. Furthermore, it has been reported by Weete(1980) that some odd-carbon chain fatty acids such as C_{11}, C_{13}, C_{15} and C_{17} have been found in fungal lipids.

When the content of lipid and fatty acids at different stages amd different parts of V. volvacea(Table 4.4) is considered, some interesting information emerges, for example: the matured stage of V. volvacea contains more lipids than its egg stage; the lipid content of the matured stage is 3.6% and that of egg stage is 2.5%. However, for the lipid content of different parts, no matter whether it is mature or egg stage, the cap's lipid is higher than that of the stem(Fig. 4.5). In the mature stage, the lipid content of the cap is 5.3% and that of the stem is 1.4%; in the egg stage, the cap's lipid is 3.5% and stem's is 1.5%. The fatty acid content of the fungus is clearly related to developmental stage and part. For example

in the mature stage, the linoleic acid content of stem makes up 82.41% of the total fatty acids but the cap only 57.42%; in the egg the values are stem 85.46% and cap 78.56%.

Table 4.2 Total Lipid and Saturated and Unsaturated Fatty Acids of Volvariella volvacea and Other Edible Species of Fungi

Sample	Total lipid (% of dry material)	Saturated fatty acids (% of total fatty acids)	Unsaturated fatty acids (% of total fatty acids
Volvariella volvacea	3.0	14.2	83.3
Lentinus edodes			
Dongko (standard grade)	2.1	18.9	76.0
Hongshin (Koshin, Hyangshin)	2.0	23.3	73.6
Kwangtung Hongko (north mushroom)	1.3	24.6	63.7
Cracky Dongko (best grade)	2.1	19.5	75.9
Agaricus bisporus	3.1	18.0	74.1
Pleurotus sajou-caju	1.6	20.0	76.6
Auricularia auricula	1.3	25.4	73.1
Tremella fuciformis	0.6	20.4	69.2

Table 4.3 Total Fatty Acid Composition of *V. volvacea* and Other Edible Species of Fuvgi

Sample	Total lipid (% of dry material)	Saponifiable material (% of lipid)	Fatty acid (% of total fatty acids) $C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Volvariella volvacea	3.0	58.8	0.48	10.50	0.62	3.47	12.74	69.91
Lentinus edodes								
Dongko (standard grade)	2.1	73.7	0.07	15.81	2.51	3.01	5.65	67.79
Hongshin (Koshin, Hyangshin)	2.0	69.3	0.71	20.94	1.81	1.66	5.23	66.53
Kwangtung Hongko (north mushroom)	1.3	73.6	0.83	20.51	3.56	3.21	6.53	53.62
Cracky Dongko (best grade)	2.1	65.0	0.13	11.31	1.88	2.07	5.27	76.25
Agaricus bisporus	3.1	68.3	0.86	11.75	1.32	5.36	3.57	69.22
Pleurotus sajou-caju	1.6	67.8	0.59	16.42	1.42	3.00	12.29	62.94
Auricularia auricula	1.3	78.1	0.69	17.30	1.12	7.35	31.60	40.39
Tremella fuciformis	0.6		0.09	17.20	2.37	3.11	38.83	27.98

$C_{14:0}$ myristic acid $C_{16:0}$ palmitic acid $C_{16:1}$ palmitoleic acid

$C_{18:0}$ stearic acid $C_{18:1}$ oleic acid $C_{18:2}$ linoleic acid

Table 4.4 Total Fatty Acid Composition of Different Stages and Different Parts of *V. volvacea*

Stage	Part	Total lipid (% of dry material)	Fatty acids (% of total fatty acids)					
			$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
matured	whole	3.6	0.48	10.25	0.62	3.47	12.74	69.91
matured	cap	5.3	0.45	9.96	0.82	6.76	21.73	57.42
matured	stem	1.4	0.35	10.54	0.40	0.80	3.76	82.41
egg	whole	2.6	0.55	8.18	0.72	1.52	4.27	82.01
egg	cap	3.5	0.48	10.01	1.23	1.82	5.61	78.56
egg	stem	1.5	0.63	6.38	0.21	1.22	2.93	85.46

$C_{14:0}$ myristic acid $C_{16:0}$ palmitic acid

$C_{16:1}$ palmitoleic acid $C_{18:0}$ stearic acid

$C_{18:1}$ oleic acid $C_{18:2}$ linoleic acid

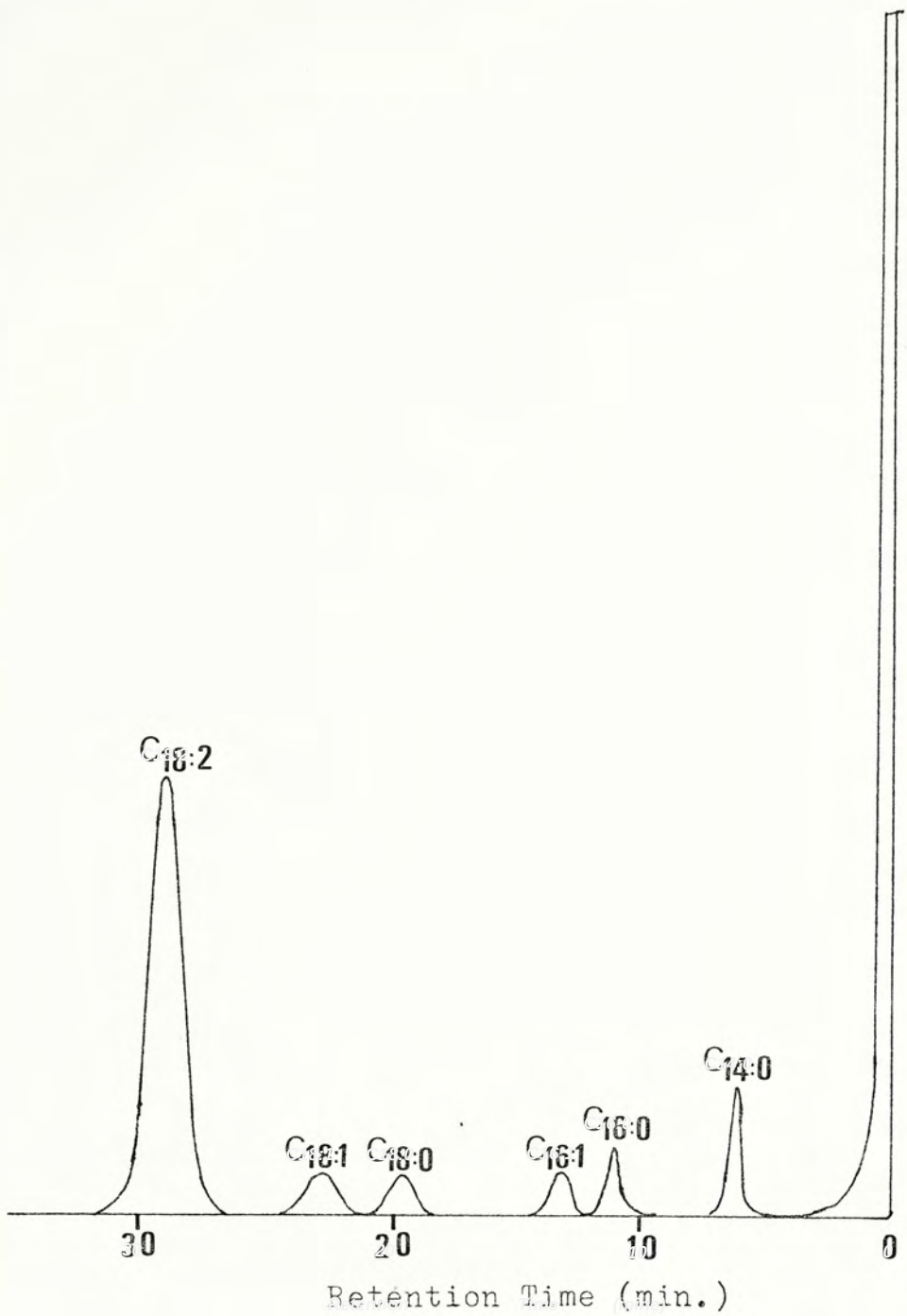


Fig. 4.2 Gas chromatogram of authentic fatty acid methyl esters

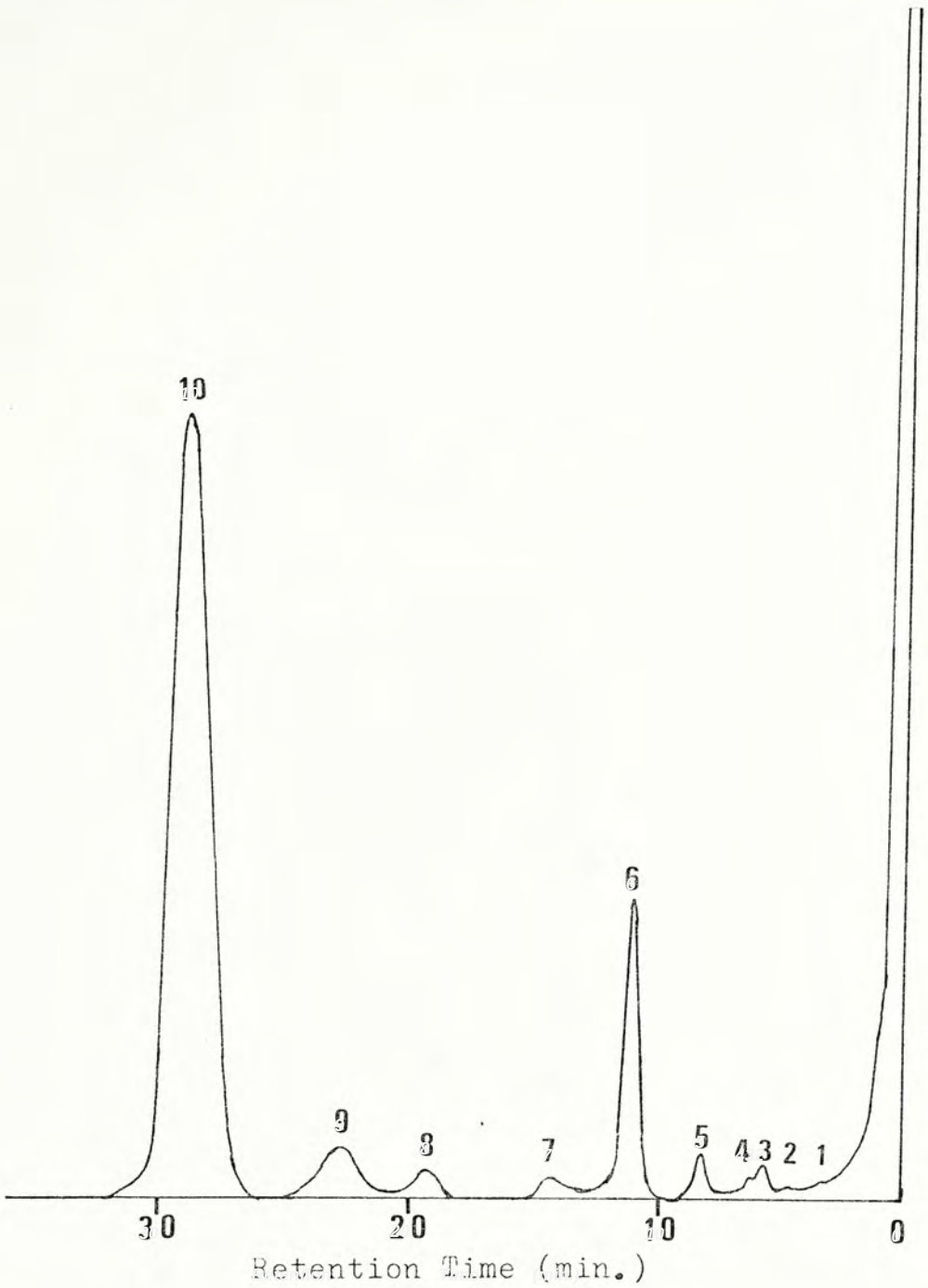


Fig. 4.3 Gas chromatogram of fatty acid methyl esters of V. volvacea

1, 2, 4, 5 unkown; 3, myristic acid

6, palmitic acid; 7, palmitoleic acid

8, stearic acid; 9, oleic acid

10, linoleic acid

Fig. 4.4 Thin layer chromatogram of fatty acid methyl esters of V. volvacea

1, authentic linoleic acid methyl ester

2, fatty acid methyl esters of V. volvacea

3, authentic oleic methyl ester

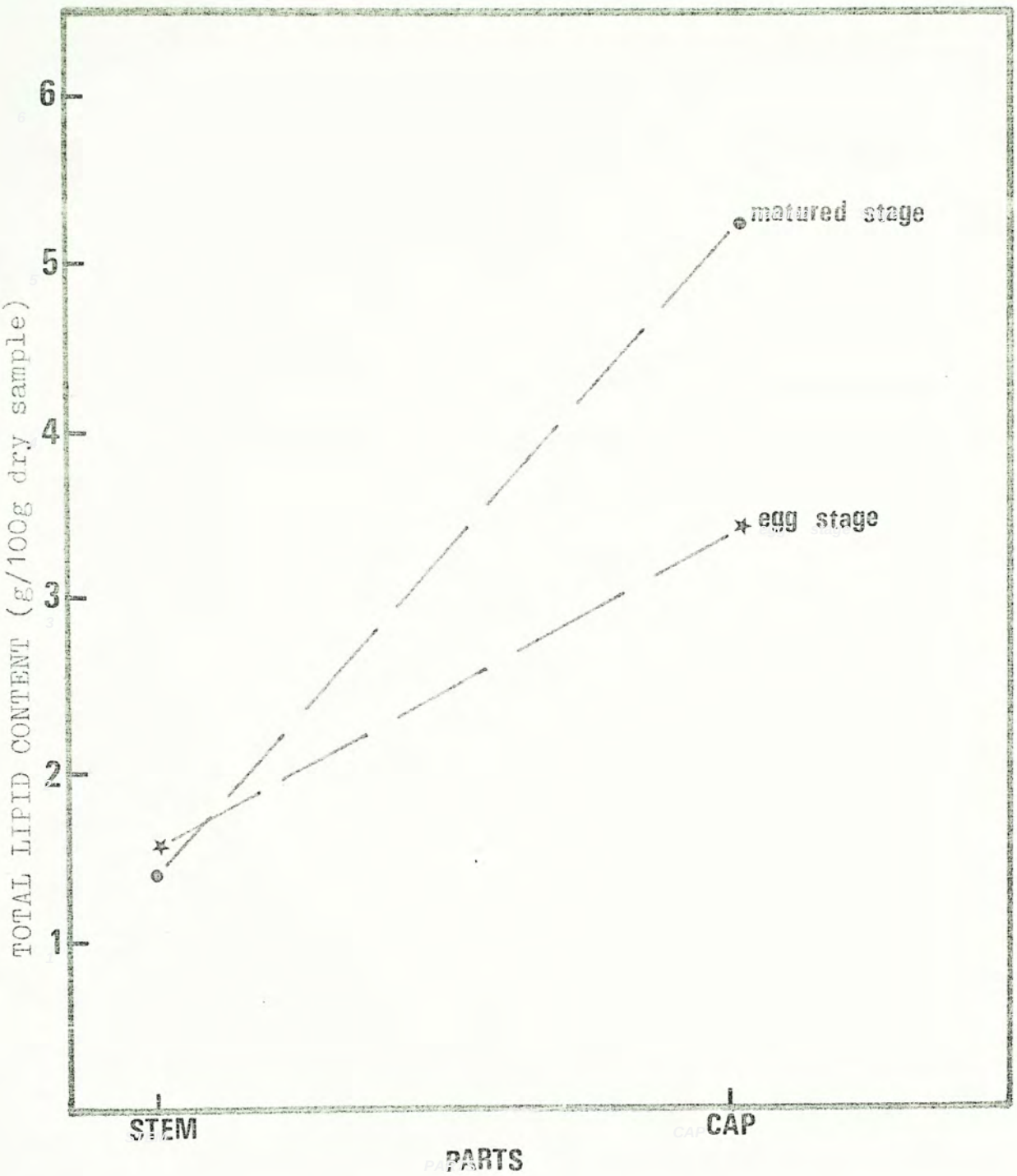


Fig. 4.5 Total Lipid Content in Different Parts of V. volvacea.

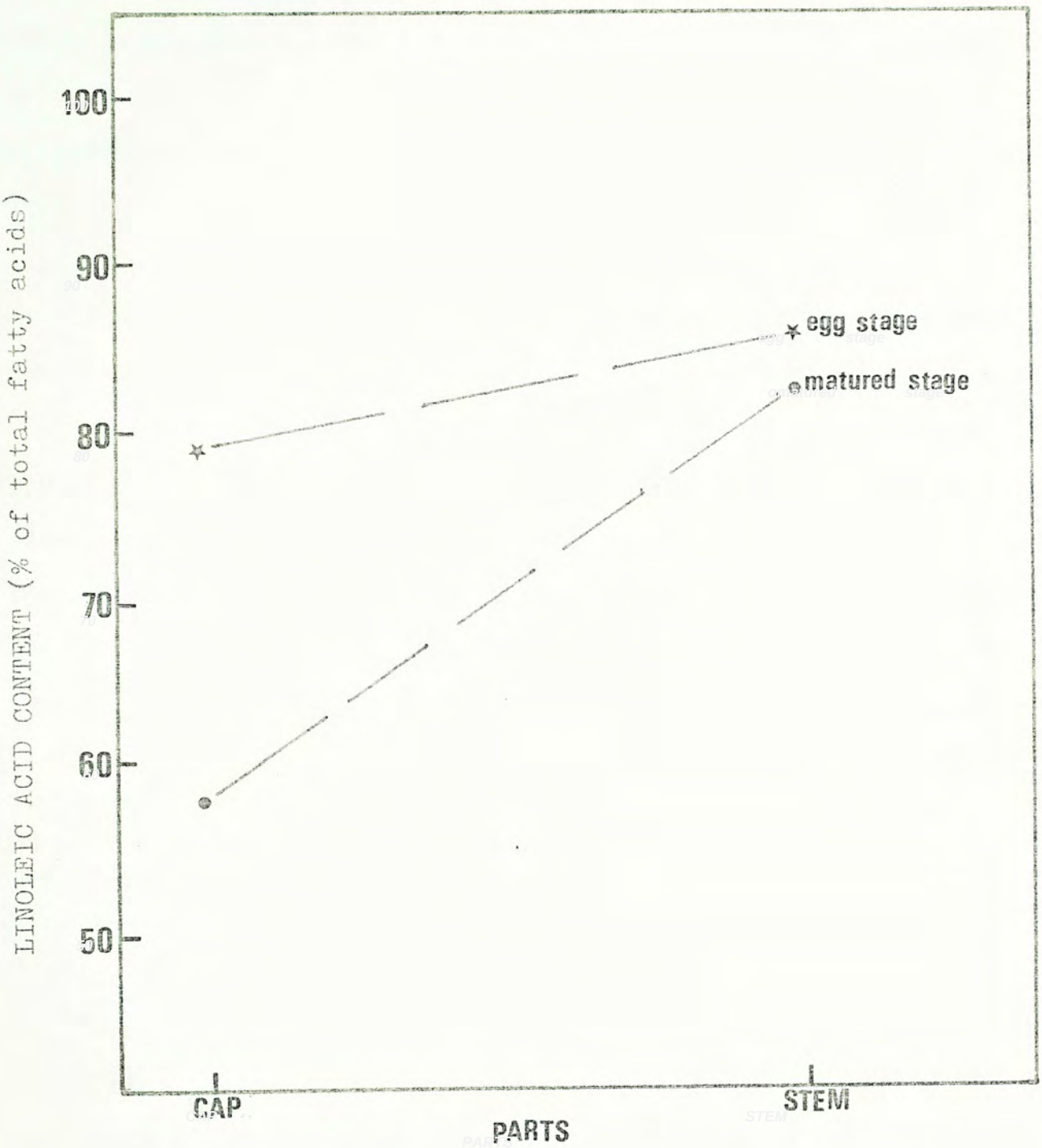


Fig. 4.6 Linoleic Acid Content in Different Parts of V. volvacea.

4.4 DISCUSSION

Since the development of GLC in the early 1950's, the fatty acid composition of numerous fungal species has been reported. Generally, fatty acids produced by fungi are similar to those of other organisms. They consist of a homologous series of saturated and unsaturated aliphatic acids ranging from 10 to 24 carbons in chain length. Without exception, even-numbered carbon chains are predominant. Fatty acids with 16 and 18 carbons are most abundant, and Palmitic is the major saturated fatty acid, and oleic and linoleic are the principal unsaturated fatty acid (Weete, 1980). Our data shown in Table 4.3 for all six edible mushrooms in general and in figures 4.7 and 4.8 for V. volvacea in particular indicate that palmitic acid and linoleic acid are also the major acids for saturated fatty acids and unsaturated fatty acids respectively. However, the stem of V. volvacea contains as 85.46% of linoleic acid, and even Weete (1980) did not mention such a high amount of linoleic acid in fungi in his comprehensive book an "Lipid Biochemistry of Fungi and Other Organisms". So, our findings are very interesting indeed.

From medical research there is growing evidence that excessive intake of saturated fatty acid in the animal fats may produce many side-effects and can cause obesity and heart disease. On the contrary, unsaturated fatty acids are required by human beings. In 1929, Burr and Burr published the

first report that fat was necessary component in the diet of rats. They showed that a low-fat diet caused poor growth, scaly skin and tail, and early death, and also that linoleic acid prevented or cured these conditions(Holman, 1976). We now know that the most obvious symptom of essential fatty acid (EFA) deficiency in animals is a scaly skin. The microscopic changes in the skin are shown in Fig. 4.9. A is the skin of a normal infant and B the skin of infant fed a milk formula containing less than 0.1% of the calories as linoleic acid. It is particularly worth noting that linoleic acid had low cholesterol content and can prevent the action of arteriosclerosis(Chu, 1980).

In contrast with some animal foods(Tables 4.3 and 4.5), edible mushrooms contain one fold or more in unsaturated fatty acid. With regard to the chief fatty acid(linoleic acid), mushrooms are higher by five times than that of animal foods. The unsaturated fatty acid content in mushroom is 74.0% to 83.5% while the linoleic acid content is 27.98% to 76.25%, but the unsaturated fatty acid of several animal foods ranges from 38.1% to 56.1% while the linoleic acid is around 7.60% to 17.20%. From the above, it can be concluded that the nutritive values of these two kinds of foods are: in protein content, the mushrooms are lower than animal foods; in the essential fatty acids and sterols, mushrooms are much higher than animal foods. In the past, data has

been lacking so that valid comparison could not be made between mushrooms and animal foods. Therefore the results of this research can help toward a complete assessment of the nutritive value of edible mushrooms.

Obviously, the resolving power of GLC is better than TLC(Fig. 4.3 and Fig. 4.4). In application of a sample, GLC can resove 10 components while TLC can resolve only 5 components. This shows that some components are overlapped in the latter case. Under our research conditions, mixed fatty acid can be separated in one GLC operation. However, we still first separate the saturated fatty acids and unsaturated fatty acids before the application GLC analysis. Such an operation gives better effect(Fig. 4.7 and Fig. 4.8) and ensures the accuracy of identification.

Table 4.5 Comparision of Fatty Acid Composition of Mushrooms with Various Foods

	Saturated fatty acid (%)	Unsaturated fatty acid (%)	Fatty acids(% of total fatty acid)						
			$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	others
V. volvacea	14.0	83.2	0.04	10.50	0.62	3.47	12.74	69.91	
L. edodes	13.5	83.5	0.13	11.31	1.88	2.07	5.27	76.25	
A. bisporus	17.9	74.0	0.86	11.75	1.32	5.36	3.57	69.22	
milk	60.9	21.8	10.5	35.9	1.1	14.5	20.6	1.2	17.3
chicken	41.9	56.1	1.2	29.9	4.5	10.8	43.6	8.0	2.0
pork	56.9	42.3	3.1	37.7	3.0	10.1	25.1	17.2	4.1
beef	48.9	38.7	1.6	28.0	2.8	19.3	38.3	7.6	12.4

Source: Kuksis(1978); Berckenridge(1978)

$C_{14:0}$ myristic acid $C_{16:0}$ palmitic acid $C_{16:1}$ palmitoleic acid

$C_{18:0}$ stearic acid $C_{18:1}$ oleic acid $C_{18:2}$ linoleic acid

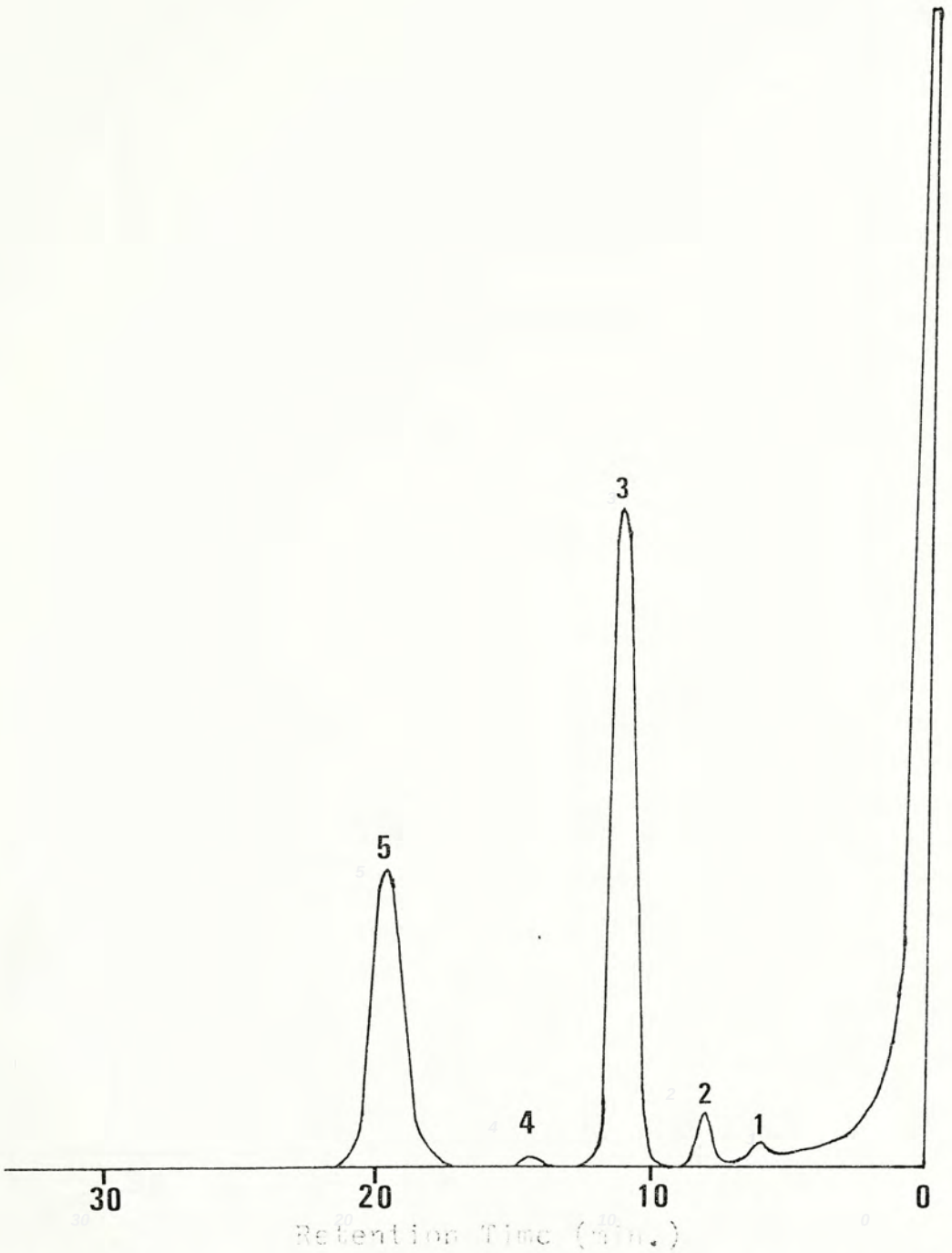


Fig. 4.7 Gas chromatogram of saturated fatty acid methyl esters of V. volvacea

Peak: 2, 4, unkown; 1, myristic acid
3, palmitic acid; 5, stearic acid

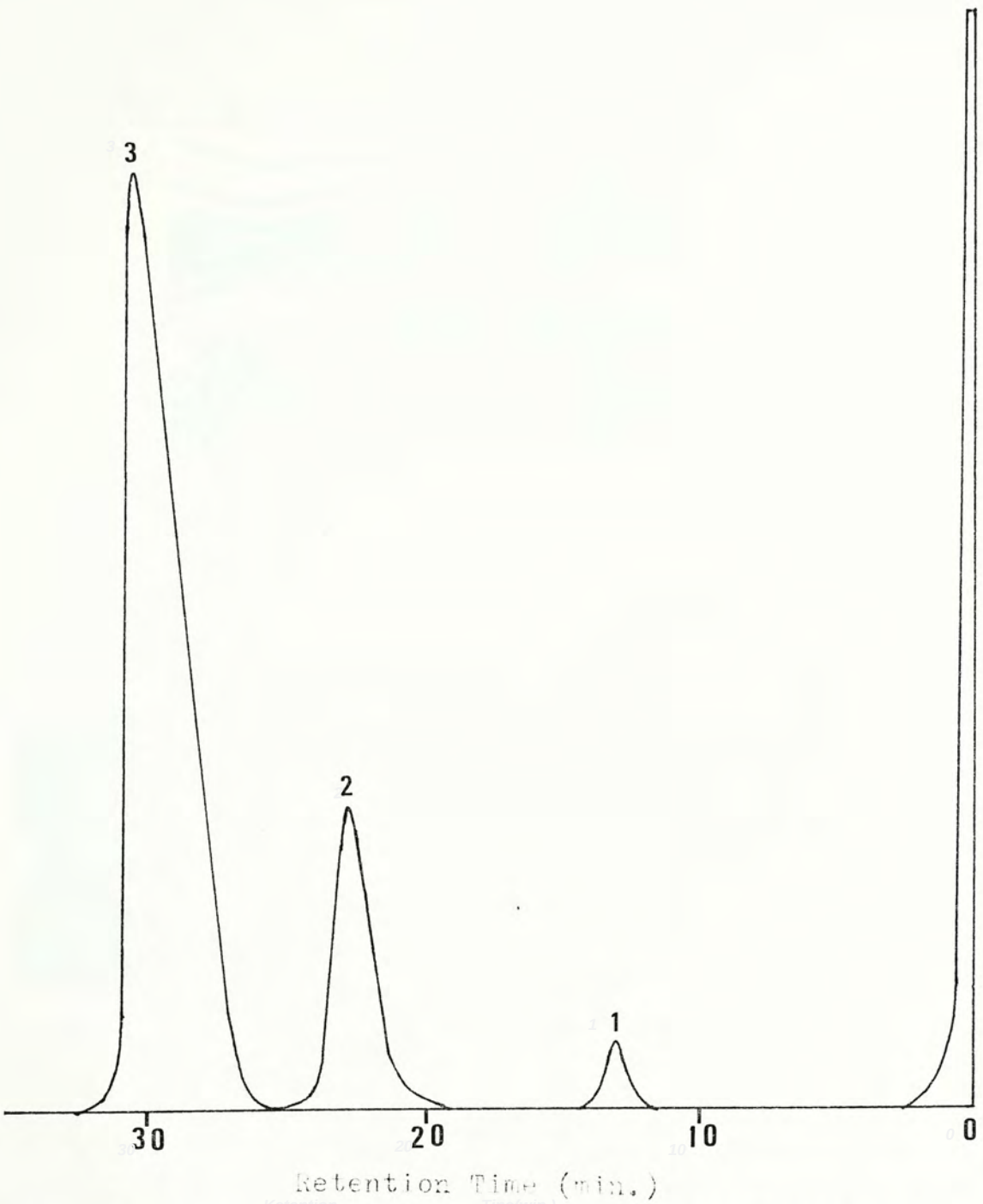


Fig. 4.8 Gas chromatogram of unsaturated fatty acid methyl esters of V. volvacea

Peak: 1, palmitoleic acid

2, oleic acid

3, linoleic acid

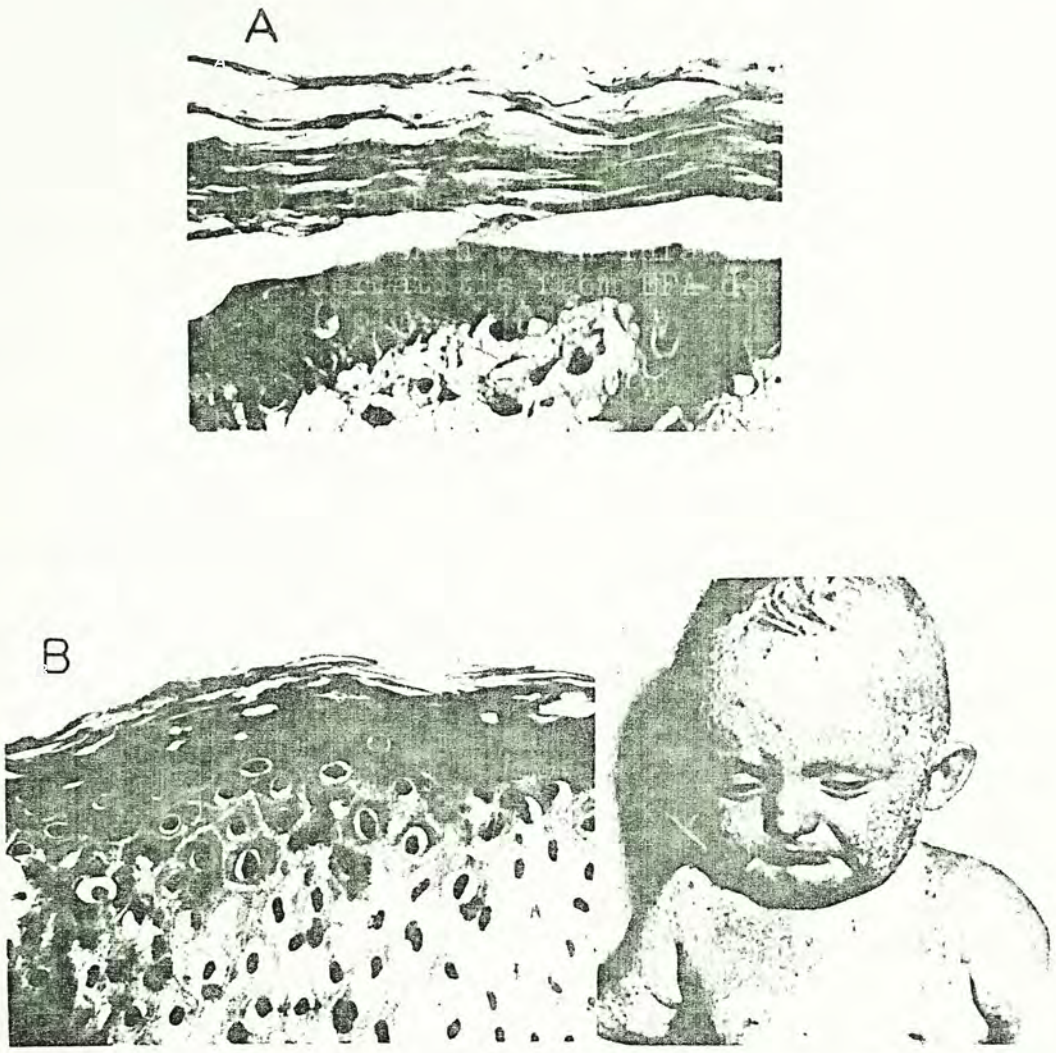


Fig. 4.9 Histologic examination of the skin of
(A) a normal infant and
(B) the skin of an infant exhibiting dermatitis from EFA deficiency.
(Holman, 1976)

Chapter 5

FLAVOUR COMPOSITION AND OTHER COMPOUNDS

5.1 DESCRIPTION OF FLAVOUR COMPOSITION

Fruiting bodies and mycelia of mushrooms always possess some flavour substances. Although people say that the flavour of mushrooms is strong, yet the content of flavour substances is low, usually measured in parts per million. The method of extracting flavour compounds is carried out usually by steam distillation or extraction with an organic solvent. The flavour compounds can be analyzed by GLC or GC-MS(Yajima, et al., 1981; Mathela and Toshi, 1981; Jacleod, et al., 1981; Papageorgiou and Argyriadou, 1981).

The extraction process of the flavour compounds and the separation of a fraction of the flavour substance in V. volvacea are described in this chapter.

5.2 MATERIAL AND METHODS

5.2.1 Extraction of flavour substance

In one method, a definite amount of material was accurately weighed and placed into a distillation flask. It was distilled with steam for 4 hours. All the distilled (comprising all the water) were collected and the mixture was extracted with diethyl ether. The ether was removed with a rotary evaporator under reduced pressure. The residue was stored in a vacuum desiccator for 12 hours and weighed. This is the flavour substance of V. volvacea.

The second method is by use of the extraction of the fat-substance as described in Section 3.2.3, which is then followed by the steam distillation method mentioned above to extract flavour substance.

5.2.2 Separation of flavour composition

As mentioned above, the flavour substance is composed of various components. A chemical method to separate the acidic fraction of the flavour substance in V. volvacea is introduced here.

A definite amount of flavour substance was dissolved in 100 ml diethyl ether and then transferred into separating funnel. 20 ml 3% sodium carbonate was added to the funnel. It was separated into two layers by shaking first and standing

until two layers were observed. The lower layer, alkaline aqueous layer, was separated. The upper layer was treated with 20 ml 3% sodium carbonate twice. The combined alkaline aqueous fraction was acidified with 0.5 N HCl until pH value reached 5-6 and then extracted with 4 x 25 ml diethyl ether. The ether solution was treated with 5% sodium bicarbonate solution three times again. The alkaline aqueous layer was acidified with dilute HCl and extracted with 4 x 25 ml diethyl ether. Finally, the ether extracts were washed with water and evaporated by a rotary evaporator under reduced pressure. A very strong flavour fraction was obtained.

5.2.3. Analysis of flavour composition by GLC

The instrument used was a Hewlett Packard 7620A Research Chromatograph. The stainless steel column (3mm x 4m) was packed with Chromosorb W AW coated with 5% Carbowax 20 M. The column temperature was programmed from 60-200°C at 4°C/min. Injection port and ionization detector temperatures were 240°C. Helium gas was used as a carrier gas at a flow rate of 40 ml/min.

5.2.4. Detection of other compounds

It was already known that V. volvacea contained protein, amino acid, carbohydrate, fat, vitamins, nucleic acid and minerals; the analysis of fatty acids and sterols have been

5.2.4.2. Detection of cardiac glycoside

The above mentioned Vb fraction was chromatographed with chloroform:acetone:ethyl alcohol (6:2:2) on silica gel TLC plate. Two pinkish red spots appeared after spraying 2% 3.5-dinitrobenzoic acid in alcohol (Kedde reagent) or 25% antimonous chloride in chloroform. These results indicated the existence of cardiac glycoside in V. volvacea.

5.2.4.3. Detection of alkaloid

Dry material of V. volvacea was extracted with 95% ethyl alcohol (containing 0.1% HCl) and then the extract was evaporated with a rotary evaporator under reduced pressure. The residue was dissolved in the dilute hydrochloric acid. The acidic extract was put in a refrigerator overnight and the supernatant was separated. The supernatant was extracted with 4 x 20 ml chloroform and then the solvent was removed by rotary evaporator under reduced pressure. An alkaline residue was obtained.

The alkaline substance was chromatographed with chloroform: methanol (8:2). Two orange coloured spots appeared by spraying Dragendorff's reagent and brown colour spots by spraying Wagner's reagent respectively (Stahl, 1969). These results indicated the existence of alkaloid in V. volvacea.

5.3 RESULTS AND DISCUSSION

5.3.1. Yield of flavour

The result of the three different ways of extraction of the flavour substance is summarized as follows:

1. From freeze-dried material, which was also used in the following two methods, the yield of flavour substance by direct steam distillation is 0.001%.
2. When first extraction with diethyl ether, then using this extracted fat-soluble substance for steam distillation, the yield is 0.0016%.
3. The fat-soluble matter was treated by sodium bicarbonate in order to extract the acidic flavour substance. The yield is 0.00054%.

5.3.2. Flavour composition by GLC

When the flavour substance was analyzed by GLC, and altogether 36 peaks (Fig. 5.1) were found. According to their RR_t, the peaks appear within 2-12 minutes probably represent 3-octenone, 3-octenol and 1-octen-3-ol. These constituents represent the common flavour substances existing in the fresh mushrooms.

Besides, there is another group of acidic flavour substances from V. volvacea. After going through the GLC analysis, it was shown that the substance consists of five compounds; one chief compound occupies a high percentage

(Fig. 5.2).

5.3.3. Detection of other compounds

The chloroform extract of V. volvacea after TLC analysis, gave an obvious triterpenoid reaction. This shows that triterpenoid is the chief component in this fraction. Nowadays, as the knowledge of medical action of various compounds increases, it is found that some triterpenoids are with anti-tumour, anti-bacterial effect and moreover, possessing the ability to lower down the blood pressure. Whether V. volvacea's triterpenoid possesses these actions needs to be confirmed.

In the methanol extract of V. volvacea, cardiac glycoside was found. This is not mentioned by any previous report. Among the various chemical compounds, cardiac glycoside is one of the chief kind of present clinically applied heart-strengthening drug. This has been appreciated by the medical science. However, the precise classification of the cardiac glycoside in V. volvacea needs further isolation and identification.

The alkaline substance of V. volvacea contains alkaloid. Before our work, Kaneda and Tokuda (1966) has isolated eritadenine from the water extract of the L. edodes. Eritadenine is kind of alkaloid which can lower down the level of the cholesterol in blood. They also proved that A. bisporus and some other edible mushrooms can reduce the concentration

of cholesterol, but nothing is mentioned about V. volvacea. Our research shows that V. volvacea contains alkaloid. However, whether this is similar to that of L. edodes which contains eritadenine needs further experimental investigation.

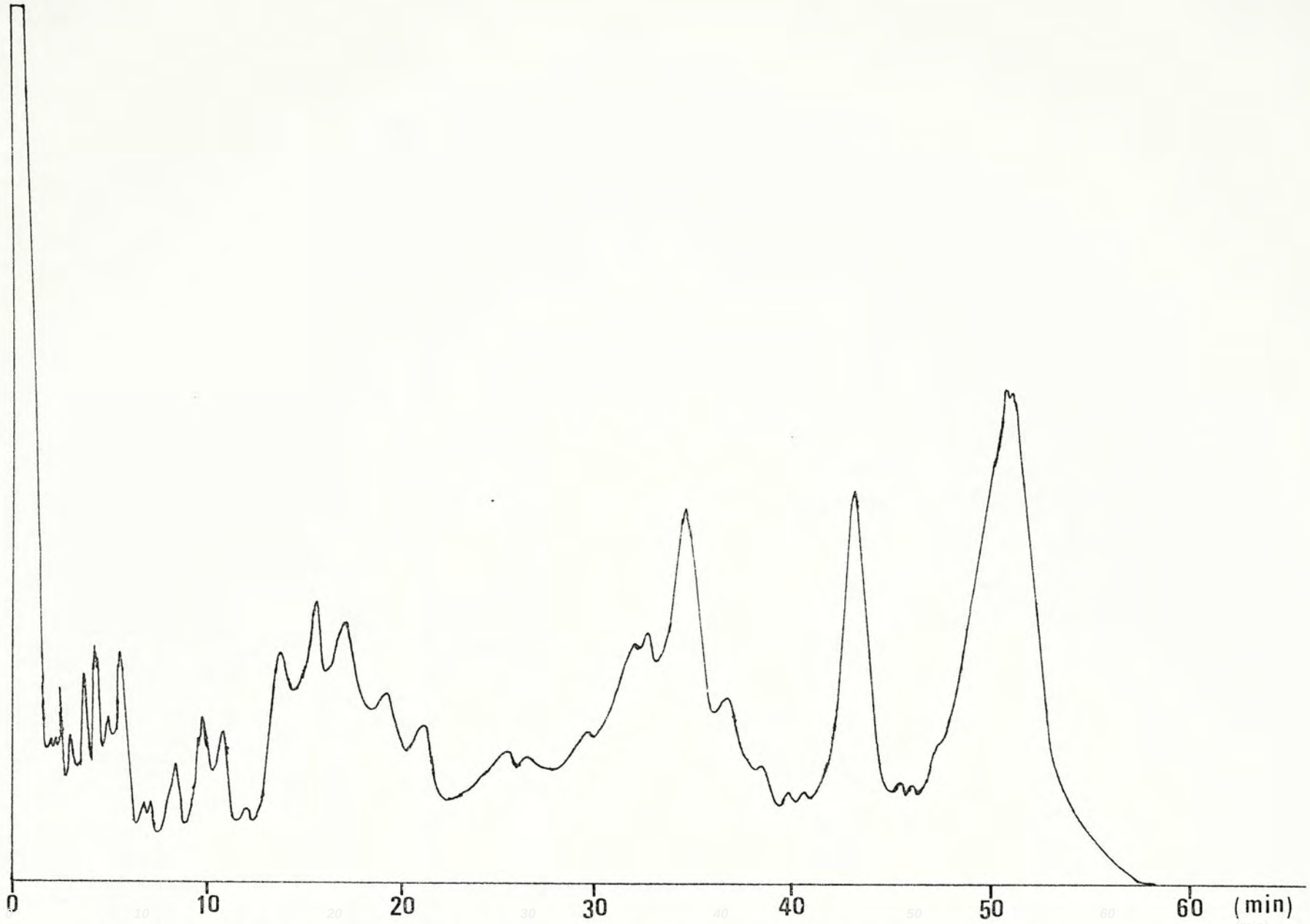


Fig. 5.1 Gas chromatogram of flavour compounds from V. volvacea

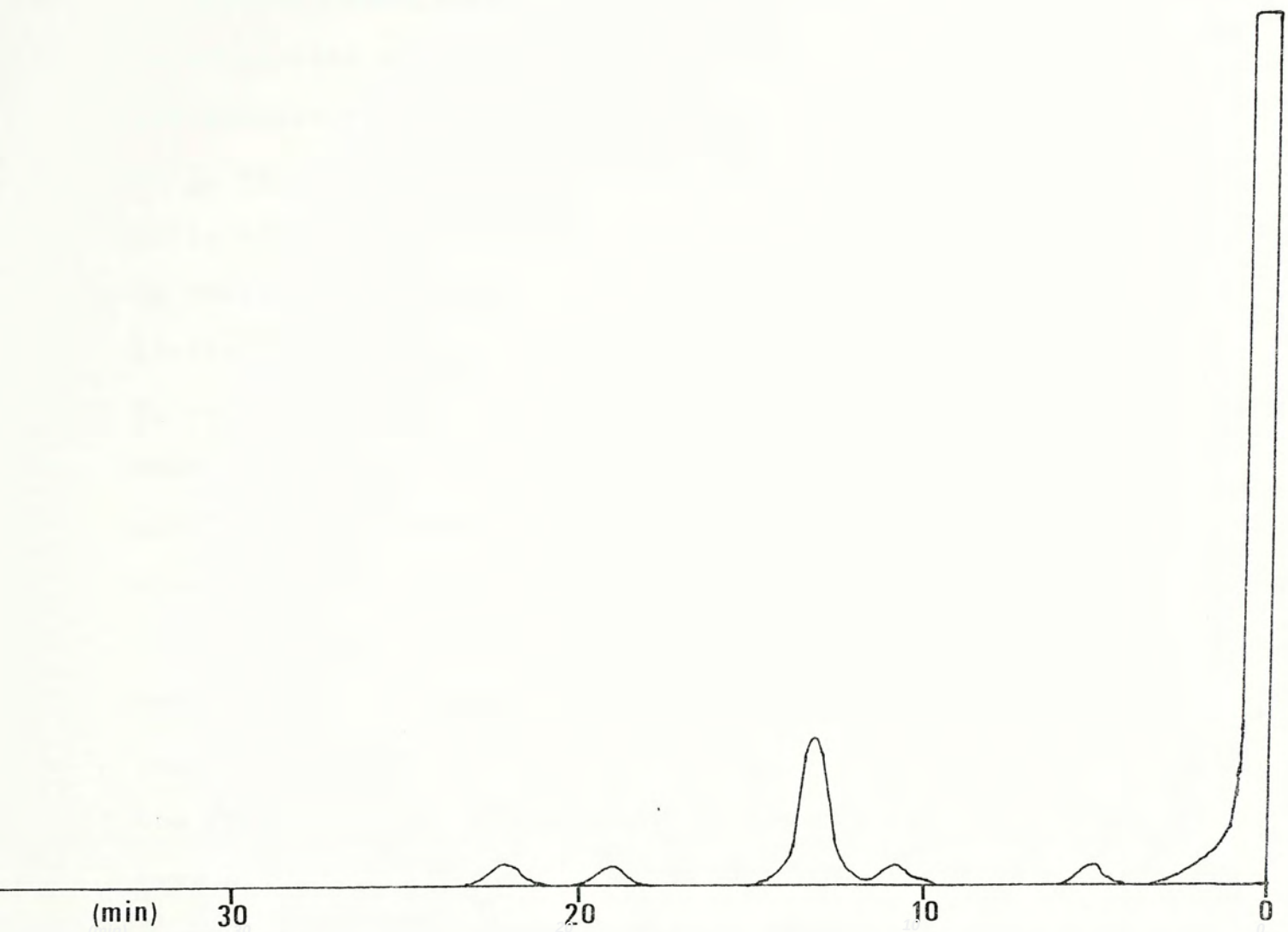


Fig. 5.2 Gas chromatogram of acidic flavour compounds from V. volvacea

Chapter 6

GENERAL SUMMARY AND CONCLUSION

Edible mushrooms have a rich content of protein, carbohydrates and vitamins. They have a particular flavour and contain biologically active substances. Mushrooms such as V. volvacea, are easily cultivated, have a short life-cycle and can utilize industrial and agricultural wastes as compost. To investigate the chemical composition of the lipids of V. volvacea, L. edodes, A. bisporus, P. sajor-caju, A. auricula and T. fuciformis was the central theme of this study. In addition, we have carried out preliminary tests of flavour substance, triterpenoid, cardiac glycoside and alkaloid in V. volvacea.

In Chapter 3, we have compared the three ways of pre-treatment of V. volvacea and two extraction methods in relation to yield of the lipid. The result showed that both the fresh material and material lyophilized after harvesting gave a much higher yield of total lipid when extracted with diethyl ether for 24 hours than did oven-dried material. The caps of the mature stage, for example, gave 5.4% and 5.9% yield of lipid respectively when using lyophilized and fresh material, but gave 3.1% only for oven-dried material.

The sterols of the lipid were separated by recrystallization and preparative TLC, and then their structures were

determinated by GLC, UV, IR, MS and NMR. The results demonstrated the presence of ergosterol in the six mushrooms. Among them, V. volvacea had the highest content (0.47%), the second was L. edodes (Cracky Dongko) (0.27%) and A. bisporus (0.23%) and T. fuciformis ergosterol was the lowest (0.01%). The ergosterol content was higher in the mature stage (0.54%) than the egg stage (0.39%) of V. volvacea. In both stages, the cap contained more ergosterol (0.63%) than the stem (0.27%). If we compare only the unsaponifiable matter, the reverse would be true; for example, the stem of the egg stage contains more ergosterol. Nowadays, it is well know that ergosterol is the precursor of vitamin D which can be formed by irradiation with an artificial light source of a fluorescent sun lamp. Since many mushrooms contain ergosterol, they can be considered as a good vitamin D source. Other than ergosterol, we had separated and identified ergosta-5,7-dien-3β-ol in L. edodes; and ergost-7-en-3β-ol in V. volvacea and T. fuciformis.

In Chapter 4, we reported that the lipid content of six edible mushrooms ranges from 0.6 to 3.1%. The highest was A. bisporus at 3.1%, the second was V. volvacea at 3.0%, and lowest was T. fuciformis at 0.6%. The mature stage of V. volvacea contained more lipids than its egg stage. The lipid content of the mature stage was 3.6% and that of egg stage was 2.5%. In the two stages of development, the caps

lipid (5.3%) was higher than that of the stem (1.4%).

Considering the saturated and unsaturated fatty acids, all six edible mushrooms analyzed had unsaturated fatty acids as their chief component. The unsaturated fatty acids of V. volvacea was as high as 83.3%, whereas that of L. edodes was 75.9%, A. bisporus 74.1%, P. sajor-caju 76.6%, A. auricula 73.1% and T. fuciformis, 69.2%. It is thus clear that the main characteristic of fatty acids in the edible mushrooms was their high content of unsaturated fatty acids. Applying GLC and TLC to the fatty acid analysis of V. volvacea and the other five species of edible mushrooms, we proved that they contain myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid. The content of linoleic acid in these edible mushrooms ranged from 27.98 to 76.25%. It is particularly worth noting that the stem of V. volvacea contained as much as 85.46% of linoleic acid. It is unusual for a single fatty acid to make up such a high proportion of the lipids in a natural product.

Linoleic acid is claimed to be a kind of essential fatty acid. Its content in the lipids of edible mushroom is higher than in animal foods by three to five times: mushrooms contains 27.98% to 76.25% of linoleic acid while animal foods contains only 7.6-17.2%. From these, we can see that though mushrooms do not have as high a fat content as the animal foods, they have a higher proportion of this essential fatty acid. This

important point should not be neglected when we compare the nutritive values of these foods.

Chapter 5 describes preliminary analyses for flavour substance, triterpenoid, cardiac glycoside and alkaloid in V. volvacea. The results show that the flavour substance of V. volvacea made up 0.001-0.0016% of its dry weight. Judging from the peaks in the GLC analysis, the flavour substance composition is very complicated. One fraction of acidic flavour substance can be separated by sodium bicarbonate solution. This acidic flavour substance of V. volvacea possesses a very strong flavour. In the chloroform extract of V. volvacea, there is a conspicuous amount of triterpenoid whereas the acidic aqueous extract contains alkaloid and the methanol extract contains cardiac glycoside. Some of them are biologically active substances. Therefore, the presence of these compounds in V. volvacea deserves forther investigation.

REFERENCES

Anderson, E. E. and C. R. Fellers. 1942. The food value of mushrooms (Agaricus campestris). Proc. Amer. Soc. Hortic. Sci. 41: 301-304.

Association of Official Analytical Chemists. 1975. "Official methods of analysis of the association of official analytical chemists (Horwitz, W., ed.)". Association of Official Analytical Chemists, Washington.

Bean, G. A. 1973. Phytosterols. In "Advance in Lipid Research II" (Paoletti, R. and D. Kritchevsky, eds.). Academic Press, New York and London.

Beily, J. P. 1981. Fatty acid and sterol composition of ungerminated spores of the Vesicular-arbuscular mycorrhizal fungus, Acaulospora laevis. Lipid. 15(11): 949-952.

Birch, G. G. 1963. Trehaloses. In "Advance in Carbohydrate Chemistry" (Wolfrom, M. L., ed.). 18: 201-225.

Birch, G. G. 1973. Mushroom sugar, Plant food for man. 1: 49-55.

Breckenridge, W. C. 1978. Stereospecific analysis of triacylglycerols. In "Handbook of Lipid Research I, Fatty Acid and Glycerides" (Kuksis, A., ed.). Plenum Press, New York and London.

Burton, R. M. 1974. Lipid extraction and separation procedure. In "Fundamental of lipid chemistry" (Burton, R. M. and F. C. Guerra, eds.). Pl-science Publications Division, Webster Groves, Missouri.

Burr, G. O. and M. M. Burr. 1929. J. Biol. Chem. 82: 345. In "Lipid 1"(Paoletti, R., G. Procellati and G. Jacini, eds.). Raven Press, New York.

Chang, S. T. 1977. The origin and early development of straw mushroom cultivation. Econ. Bot. 31: 374-376.

Chang, S. T. 1981. Mushrooms and mushroom science. Mushroom Newsletter for the Tropics 1(3): 2-6.

Chang, S. T. and W. A. Hayes.(eds.) 1978. "The Biology and Cultivation of Edible Mushrooms". Academic Press, New York and London.

Chang, S. T. and T. H. Quimio.(eds.) 1982. "Tropical Mushrooms: Biological Nature and Cultivation Methods." The Chinese University Press, Hong Kong.

Cheng, S. C. 1979. Study on the nutritive value of the straw mushroom, Volvariella volvacea.(M. Phil. thesis, Division of Biology, The Chinese University of Hong Kong).

Chihara, G., J. Hamuro, Y. Y. Maeda. Y. Arai and F. Fukuoka. 1970. Antitumour polysaccharide derived chemically from natural glucan(Pachyman). Nature 225: 943-944.

Chihara, G., Y. Maeda, J. Hamuro, T. Sasaki and F. Fukuoka. 1969. Inhibition of mouse Sarcoma-180 by polysaccharide from Lentinus edodes(Berk.) Sing. Nature 222: 687-688.

Chu, T. P. 1980. Utilization of plant oil. Botany J. 10: 6-7.

Chung, H. S., B. K. Kim, K. S. Chung and M. E. Yang. 1980. Studies on the antineoplastic components of Korea Basidiomycetes. Kor. J. Mycol. 8(2): 107-113.

Clark, J. M. 1964. "Experimental Biochemistry." W. H. Freeman

and Company, San Francisco and London.

Cochran, K. W., N. R. Goulet and C. C. Brown. 1960. Differential and specific inhibition of ECHO viruses by plant extracts. Proc. Soc. Exp. Biol. and Med. 103: 96.

Cook, C. H. and S. H. An. 1975. Studies on the components in the ethereal extract of Panax ginseng (I). Kor. J. Pharmacogn. 6: 15.

Creech, H. J. and E. Breuninger. 1962. Polysaccharide-peptide complex from Serratia marcescens cell. Can. J. Biochem. 42: 593-603.

Crisan, E. V. and A. Sands. 1978. Nutritional value. In "The Biology and Cultivation of Edible Mushrooms" (Chang, S. T. and W. A. Hayes, eds.). Academic Press, New York and London.

Daniel, J. R. and C. S. Lee. 1981. Mushrooms consumption, production and culture development. Mushroom News 29 (7): 25-36.

Edwards, R. L. 1975. The food value of the mushroom. Mushroom J. 31: 228-233.

Flegg, P. B. and G. A. Maw. 1976. Mushrooms and their possible contribution to world protein needs. Mushroom J. 48: 396-405.

Gen, Y. 1977. The flavour and medicinal activity of edible mushrooms. Food Science Abstracts. 4(10): 52-58.

Gilbert, F. A. and R. F. Robinson. 1975. Food from fungi.

Econ. Bot. 11: 126-145.

Gruen, H. E. 1964. Mushroom growing in Japan. Boston Mycol. Club, Bull. No. 1.

Haddad, N. A. and W. A. Hayes. 1979. Nutritional factors and the composition of Agaricus bisporus mycelium. Mushroom Science X (Part 2): 715-722.

Hamuko, J., Y. Yamashita, Y. Ohsaka, Y. Y. Maeda and G. Chihara. 1971. Carboxymethylpachymaran, A new water soluble polysaccharide with market antitumor activity. Nature 223: 486-487.

Hatamaka, S. I., Y. Mimura, K. Taniguchi, G. Kinoshita and H. Katayama. 1974. Specific amino acids in some edible mushrooms. Mushroom Science IX (Part 1): 809-814.

Holman, R. T. 1976. Significance of essential fatty acids in human nutrition. In "Lipid 1" (Paoletti, R., G. Procellati and G. Jacini, eds.). Haven Press, New York.

Holtz, R. B. and D. E. Smith. 1979. Lipid metabolism of mushroom mycelia. Mushroom Science X (Part 1): 437-444.

Horning, E. C., C. J. W. Brooks and W. J. A. Vanedn Heuvel. 1968. Gas phase analytical methods for the study of steroids. In "Advance in Lipid Research 6" (Paoletti, R. and Kritchevsky, D. eds.). Academic Press, New York and London.

Hughes, D. H., D. L. Lynch and G. F. Somers. 1958. Chromatographic identification of the amino acids and

carbohydrates in the cultivated mushroom Agaricus campestris L. ex Fries. J. Agric. Food Chem. 6: 850-853.

Humfeld, H. 1948. The production of mushroom mycelium Agaricus campestris in submerged culture. Mushroom Science IX (Part 1): 809.

Jacleod, A. J., N. M. Pieris and V. Gil. 1981. Volatile components of sugar beet leaves. Phytochemistry 20(9): 2292-2295.

Jandaik, C. L., A. R. Bhandari, C. L. Arora and C. O. Rangdad. 1979. Chemical composition of some edible fungi. Mushroom Science X (Part 2): 685-688.

Kamiya, T., Y. Saito, M. Hashimoto and H. Seki. 1972. Hypocholeterolemic alkaloids of Lentinus edodes (Berk) Sing. I, Structure and synthesis of eritadenine. Tetrahedron 28: 899-906.

Kaneda, T. and S. Tokuda. 1966. Effect of various mushrooms preparation on cholesterol levels in rats. The Journal of Nutrition 90(4): 371-375.

Kaneda, T., K. Arai and S. Tokuda. 1963. The effect of dried mushroom, Cortinellus shitaka on cholesterol metabolis in rats (I). 栄養と食糧 16: 106-108.

Khanna, P. and H. S. Garcha. 1981. Nutritive value of mushroom Pleurotus florida. Mushroom Science XI (Part 2): 561-572.

Kim, B. K., E. K. Park and M. J. Shim. 1979. Studies on the constituents of higher fungi of Korea (XXIII), Anti-

neoplastic activities of Coriolus versicolor (L. ex Fr.) Quel, Pleurotus ostreatus (Fr.) Kummer and Lentinus edodes (Berk.) Sing. Arch. Pharm. Rec. 2(2): 145-151.

Kuksis, A. 1978. Separation and determination of structure of fatty acids. In "Handbook of Lipid Research 1, Fatty Acid and Glycerides". Plenum Press, New York and London.

Kurtzman, R. H. 1975. Mushroom as a source of food protein. In "Protein Nutritional Quality of Foods and Feeds" (Friedman, M. ed.). Marcel Dekker, Inc., New York.

Lisboa, B. P. 1974. Identification of sterols and steroids. In "Fundamentals of Lipid Chemistry" (Burton, R. M. and F. C. Guerra, eds.). Bi-Science Publication Division, Webster Groves, Missouri.

Littlewood, A. R. 1970. Gas Chromatography, Principles, Techniques, and Applications. Academic Press, New York and London.

Maeda, Y. Y., J. Hamuro and G. Chihara. 1971. The mechanisms of action of antitumour polysaccharide, I. The effect of antilymphocyte serum on the antitumour activity of lentinan. Int. J. Cancer 8: 41-46.

Mangold, H. K. 1961. Thin layer chromatography of lipids. J. American Oil Chemists Society 38: 708-727.

Mangold, H. K. 1969. Aliphatic lipids. In "Thin Layer Chromatography" (Stahl, E. ed.). Springer-verlag Berlin, Heidelberg, New York.

Mathela, C. S. and P. Joshi. 1981. Terpene from the essential oil of Cymbopogon distans. Phytochemistry 20(12): 2770-2771.

McConnell, J. E. W. and W. B. Esselen Jr. 1974. Carbohydrates in cultivated mushroom Agaricus campestris. Food Res. 12: 118-121.

Morita, K. and S. Kobayashi. 1966. Isolation and synthesis of lenthionine, an odorous substance of shiitake, an edible mushroom. Tetrahedron letters 6: 573-577.

Ogundana, S. K. and O. Fagade. 1981. The nutritive value of some Nigerian edible mushrooms. Mushroom Science XI (Part 1): 123-131.

Oka, Y., S. Kiriyama and A. Yoshida. 1973. Sterol composition of fruits, fungi, marine algae, tea, coffee, and cocoa. Eiyo to Shokuryo. 26: 317-327 (in Japanese) (Food Sci. Technol. Abstr. 6: 47).

Ono, T., K. Arimoto, K. Kano, K. Matsuoka, W. Sugiura, H. Sadone and K. Mori. 1974. Vitamin D_2 formation in Lentinus edodes (Shii-ta-ke) by irradiation with a flourescent sunlamp. Mushroom Science IX (Part 1): 435-443.

Quimio, T. H. 1981. Nutritional studies on Volvariella volvacea. Mushroom Newsletter for the Tropics 2(1): 9-13.

Oura, H. and S. Nakashima. 1972. Effect of radix ginseng extract on serum protein synthesis. Chem. Pharm. Bull. 20(5): 980-986.

Papageorgiou, V. P. and Argyriadou. 1981. Trace constituents in the essential oil of Thymus capitatus. Phytochemistry 20(9): 2295-2297.

Parent, G. and D. Thoen. 1978. Consideration sur la teneur en proteines et en acides gras de quelques especes de champignons comestibles du Shaba (Zaire). Mushroom Science X (Part 2): 689-693.

Park, E. K., E. C. Choi and B. K. Kim. 1979. Studies on the constituents of higher fungi of Korea (XVIV), chemical analysis of antineoplastic components of Coriolus versicolor (L. ex Fr.) uel., Pleurotus ostreatus (Fr.) Kummer and Lentinus edodes (Berk.) Sing. Arch. Pharm. Res. 2(2): 153-157.

Park, Y. H., H. G. Chang, C. S. Jung and D. S. Kim. 1974. Some experiments on the cultivation of straw mushroom, Volvariella volvacea (Pull. ex. Fr.) Sing. in Korea. Korean J. Mycol. 2: 21-24.

Perkins, E. G. 1975. Gas chromatography-mass spectrometry of lipids. In "Analysis of Lipids and Lipoproteins". American Chemist's Society-Champaign, Illinois.

Pyysalo, H. 1979. On the formation of the aroma of some northern mushroom. Mushroom Science X (Part 2): 669-684.

Quimio, T. H. 1981. Nutritional studies on Volvariella volvacea. Mushroom Newsletter for the Tropics 2(1): 9-13.

Roe, E. M. F. 1959. Growth inhibition of mouse ascites tumor cell by powdered tragacanth. Nature 184: 1891.

Ruseva-Atanasona, N. and J. Janak. 1966. Multidimensional chromatography using different developing methods, IV.

Rational identification of fatty acid esters. J. Chromatog. 21: 207.

Saito, Y., M. Hashimoto, H. Seki and T. Kamiya. 1970. Two new constituents from Lentinus edodes. Tetrahedron letters 56: 4863-4866.

Sakai, S., G. Saito, J. Sugayama, T. Takano and S. Takeda. 1964. Anticancer effect of polysaccharide fraction prepared from bamboo grass. Gann. 55: 197-203.

Samajpati, N. 1979. Nutritive value of some Indian edible mushrooms. Mushroom Science X (Part 2): 695-703.

Sasaki, T,, Y. Arai, T. Ikekawa, G. Chihara and F. Fukuoka. 1971. Antitumour polysaccharide some polyporaceae, Ganoderma applanatum (Pers.) Pat and Phellinus linteus (Berk. et Curt) Aoshima. Chem. Pharm. Bull. 19(4): 821-826.

Shanghai Institute of Materia Medica, Academia Sinica. 1972. Extraction and separation of active constituent of Chinese herbal medicine. Shanghai Science and Technology Publishing House.

Stahl, E. 1969. Thin Layer Chromatography. Springer-Verlag, Berlin, Heidelberg, New York.

Sumi, M. 1933. On the ergosterin content of various edible mushroom in Japan. Sci. Pap. Inst. Phys. Chem. Res. 20: 524-528 (in Japanese).

Suzuki, F., T. Suganuma and N. Is ida. 1974. Interferon

induction by an antitumour antibiotic, lymphomycin. Journal of Antibiotics 27(5): 346-348.

Tokita, F., N. Shibukawa, T. yasumoto and T. Kaneda. 1971. Effect of mushroom on cholesterol metabolism in rats (VI) separation and chemical structure of the plasma cholesterol reducing substance from mushroom. 栄養と食糧 24(2): 92-95.

Tokuda, S., and T. Kaneda. 1964. Effect of dried mushroom, Lentinus edodes (Berk.) Sing. on cholesterol metabolism in rats (II). 栄養と食糧 17: 73-76.

Tokuda, S., Y. Sugawara and T. Kaneda. 1973. Effect of mushroom on cholesterol metabolism in rats (IX), reducing mechanism of plasma cholesterol by mushroom (III). 栄養と食糧 26(2): 113-119.

Tokuda, S., A. Tagiri, E. Kano and T. Kaneda. 1971. Reducing mechanism of plasma cholesterol by mushroom, effect of mushroom on cholesterol metabolism in rats (VII) 栄養と食糧 24(9): 477-480.

Tokuda, S., A. Tagiri, E. Kano, Y. Sugawara, S. Suzuki, H. Sato and T. Kaneda. 1974. Reducing mechanism of plasma cholesterol by Shii-ta-ke. Mushroom Science IX (part 1): 445-461.

Torey, A. 1968. Submerged culture of higher fungi mycelium on an industrial scale. Mushroom Science VII: 585-589.

Tsumoda, A. and N. Ishida. 1970. A mushroom extract as an

interferon inducer. Annals of the New York Academy of Science 173: 719-726.

Vicenio, A. S. 1961. A study on mushroom culture in the Philippines. Philipp. Agric. Forester. 5: 119.

Wang, Y. Z. 1981. Congratulation on the open publication of edible fungi. Edible Fungi 1: 1.

Weete, J. D. and J. W. Darrell. 1980. "Lipid Biochemistry of Fungi and Other Organisms" Plenum Press. New York and London.

Wijngaarden, D. Van. 1967. Modified rapid preparation of fatty acid ester from lipids for gas chromatographic analysis. Anal. Chem. 39: 848.

Yajima, I., T. Yanai, M. Nakamura, H. Sakakibara and K. Hayashi. 1981. Volatile flavour compounds of matsutake-Tricholomam atsutake(Ito et Imai) Sing. Agric. Biol. Chem. 45(2): 373-377.

Yang, Y. P. and Y. D. Chao. 1981. Pharmacopoeia(Sinica). Hei Lun-jang Science and Technology Publishing House.

Yang, C. R. 1981. The biological basis of edible fungi. Shanghai Science and Technology Publishing House.

Yokokawa, H.and T. Mitsugashi. 1981. The sterol composition of mushroom. Phytochemistry 20(6): 1349-1351.

Yoshida, H., J. Hayashi, Y. Aoyagi and T. Sugahara. 1979. Fatty acid composition and ergosterol contents of different grades of dried Lentinus edodes(Berk.) Sing.

Nippon Shokuhin Kogyo Gakkaishi. 26(5): 221-224.

Yu, J. G. 1981. Studies on chemical constituents of Ganoderma capense(Lloyd) Teng (II). Chinese medicinal herbs 12(7): 7-11.